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 formulation having negative stabilizer, Phosphatidyl glycerol shown minimum % free drug, optimum particle size and least percent drug release when compared to the formulations having positive stabilizer, Stearylamine. The release kinetics of formulations containing neural, negative and positive stabilizers followed zero-order release kinetics. Hence it could be concluded that stabilizes like Stearylamine and Phosphatidyl 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, Phosphatidyl glycerol, Ammonium sulphate.

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 makes up cell membrane [1].

Doxorubicin hydrochloride (C$_\text{22}$H$_\text{18}$N$_\text{2}$O$_\text{8}$) 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 Myelosupression [2].

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 into tumor tissue [3,4].

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 [5].

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 invitro release studies.

MATERIALS AND METHODS
Materials
Doxorubicin hydrochloride, Hydrogenated Soy Phosphatidyl Choline and Cholesterol were obtained as a sample from Celon labs Hyderabad. Stearylamine and Phosphatidyl glycerol were obtained from Sigma Aldrich. The ingredients were analytical grade. The laboratory grade chemicals used for the work are Ammonium sulphate, Sacrose, 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 phosphates 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[6].

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 Phosphatidyl glycerol are dissolved in chloroform and rotated in a rotavapor by applying vacuum of about 25mmHg at 25°C; 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 [7,8].

In-process Checks

RPM: 50 rpm (Film formation), 70 rpm (Hydration), 60 rpm (Drug Loading).

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

Physical characterization of liposomes [9,10,11]

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.

Percent free drug

Absorbance of solution was measured at 590 nm using sucrose Histidine solution as blank. 0.1ml of sample was transferred into 20 ml stopped test tube, add 8ml of Sucrose-Histidine solution to it, mix well. Absorbance was measured at 590 nm using calibrated UV spectrophotometer. Transferred the solution from the cell to test tube (A). To the above test tube containing solution, added 1 ml sodium hydroxide solution, mix well and absorbance was measured at 590 nm using UV transfer the solution from the cell to test tube (A). To the above test tube containing solution, added 1 ml of Triton X-100 solution, mixed well and measured the absorbance at 590 nm using calibrated UV (A).

Percent Free Doxorubicin HCl = [(A₁ x 1.125) – A₀ x 1.25] x 100 [12]

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 x W/200 x 5/50 x C/100 x 100/D x 100 x 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 [12].

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 200 ml of PBS (pH 7.4), maintained at 37°C and stirred with the help of a magnetic stirrer. Aliquots (4 ml) 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 λ max of 254 nm [13].

Release kinetics

To analyze the in vitro release data various kinetic models were used 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 [14,15].

a. Zero order kinetics

Zero order release would be predicted by the following equation:

\[ A_t = A_0 - K_t t \]

When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys Zero – order kinetics and its slope is equal to Zero order release constant K₀.

b. First order kinetics

First order release could be predicted by the following equation:

\[ \log C = \log C_0 - K_t t / 2.303 \]

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 ‘K’ can be obtained by multiplying 2.303 with the slope value.

c. Higuchi’s model

Drug release from the matrix devices by diffusion has been described by following Higuchi’s classical diffusion equation.

\[ Q = D t (\pi / 12) C_s (2t^2 - 3t^2) / t^2 \]

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 released by diffusion mechanism. The slope is equal to ‘K’ (Higuchi’s 1963).

d. Korsmeyer equation / Peppa’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/ Peppa’s law equation) mentioned in below table 1, which is often used to describe the drug release behavior from polymeric systems.

\[ M_t / M_0 = K t^n \]

<table>
<thead>
<tr>
<th>S. No</th>
<th>Diffusion Exponent (n) Overall solute diffusion mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.45</td>
</tr>
<tr>
<td>2.</td>
<td>0.45&lt;n&lt;0.89</td>
</tr>
<tr>
<td>3.</td>
<td>0.89</td>
</tr>
<tr>
<td>4.</td>
<td>n=0.89</td>
</tr>
</tbody>
</table>

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 λ max 254 nm. The linearity was plotted for absorbance against concentration with R² value 0.9995 and with the slope equation y=0.0179x-0.003. The absorbance values and standard curve shown in Fig 1.
Fig. 1: Standard graph of Doxorubicin hydrochloride in phosphate buffer of pH 7.4.

Fig 2: FTIR spectrum of Doxorubicin HCl

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.

Fig 3: FTIR spectrum of Doxorubicin HCl liposome injection

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
The composition and ratios of compounds showed in Table 2. Among those compositions, 9 formulations are selected as optimized batches for further evaluation, 3 from each of neutral, positive, and negative as shown in Table 3.

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

<table>
<thead>
<tr>
<th>Ratio of ingredients</th>
<th>Types of liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPC : cholesterol : Stearyl amine : Phosphatidyl glycerol : Ammonium Sulphate</td>
<td>Neutral</td>
</tr>
<tr>
<td>5:5:0:0:30</td>
<td>5:5:1:0:30</td>
</tr>
<tr>
<td>5.5:4.5:5.0:0:30</td>
<td>5.5:5.5:1:0:30</td>
</tr>
<tr>
<td>6.5:5.5:5.0:0:30</td>
<td>6.5:6.5:1:0:30</td>
</tr>
<tr>
<td>7:6.5:6.0:0:30</td>
<td>7:7.0:1:0:30</td>
</tr>
<tr>
<td>7.5:7.0:7.0:0:30</td>
<td>7.5:7.5:1:0:30</td>
</tr>
<tr>
<td>8:8.0:8.0:0:30</td>
<td>8:8.5:1:0:30</td>
</tr>
<tr>
<td>8.5:8.5:8.0:0:30</td>
<td>8.5:9.0:1:0:30</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Drug (mg/ml)</th>
<th>Hydrogenated Soy Phosphatidyl Choline (mg/ml)</th>
<th>Cholesterol (mg/ml)</th>
<th>Stearylamine (mg/ml)</th>
<th>Phosphatidyl glycerol (mg/ml)</th>
<th>Ammonium Sulphate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F2</td>
<td>2</td>
<td>7.5</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F3</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F4</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F5</td>
<td>2</td>
<td>7.5</td>
<td>2.5</td>
<td>1</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F6</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>F7</td>
<td>2</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>F8</td>
<td>2</td>
<td>7.5</td>
<td>2.5</td>
<td>-</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>F9</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>

### 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 4 and 5.

#### 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 F1 to 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.

#### Assay

The assay value is determined for all the formulations from F1 to 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 release of F1 to F9 formulations at various time intervals was calculated. The cumulative percent drug release in F3, F6, F9 formulations was plotted against time in Figure 6. 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-Peppa’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 7, 8, 9 & 10 and in Table 6.

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

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulation code</th>
<th>Average vesicular size (nm)</th>
<th>Zeta Potential(mV)</th>
<th>Poly dispersive index (Pdi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>F3</td>
<td>333nm</td>
<td>4.31</td>
<td>0.652</td>
</tr>
<tr>
<td>2.</td>
<td>F6</td>
<td>556nm</td>
<td>23.68</td>
<td>0.782</td>
</tr>
<tr>
<td>3.</td>
<td>F9</td>
<td>317nm</td>
<td>-23.4</td>
<td>0.645</td>
</tr>
</tbody>
</table>
Table 5: % free drug and Assay of F1, F2, F3, F4, F5, F6, F7, F8 and F9 formulations

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulation code</th>
<th>Percentage of free drug</th>
<th>Doxorubicin Hcl Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>F1</td>
<td>14.26±2.3%</td>
<td>100.8±0.11%</td>
</tr>
<tr>
<td>2.</td>
<td>F2</td>
<td>13.36±1.1%</td>
<td>102.8±0.32%</td>
</tr>
<tr>
<td>3.</td>
<td>F3</td>
<td>12.36±1.1%</td>
<td>101.7±0.65%</td>
</tr>
<tr>
<td>4.</td>
<td>F4</td>
<td>12.98±5.6</td>
<td>104.3±0.42%</td>
</tr>
<tr>
<td>5.</td>
<td>F5</td>
<td>12.56±4.6</td>
<td>104.6±0.65%</td>
</tr>
<tr>
<td>6.</td>
<td>F6</td>
<td>11.36±1.3%</td>
<td>103.3±0.96%</td>
</tr>
<tr>
<td>7.</td>
<td>F7</td>
<td>9.13±1.2%</td>
<td>104.25±0.38%</td>
</tr>
<tr>
<td>8.</td>
<td>F8</td>
<td>8.45±5.9%</td>
<td>105±0.58%</td>
</tr>
<tr>
<td>9.</td>
<td>F9</td>
<td>6.83±0.8%</td>
<td>109±1.32%</td>
</tr>
</tbody>
</table>

Fig. 4: SEM of F6 formulation
Fig. 5: SEM of F9 formulation

Fig. 6: Comparison of in vitro release studies for optimized formulations F3, F6, F9

Fig. 7: Zero order release studies for optimized formulations F3, F6 & F9

Fig. 8: First order release studies for optimized formulations F3, F6 & F9
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 7 & 8.

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.

REFERENCES