FORMULATION AND CHARACTERIZATION OF DOXORUBICIN HYDROCHLORIDE LIPOSOMES BY DOUBLE EMULSION METHOD

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ABSTRACT

Doxorubicin hydrochloride is one of the most commonly used cytotoxic anthracycline antibiotics used in cancer chemotherapy and has been shown to have activity against a wide variety of neoplasms. Conventional compositions of doxorubicin hydrochloride are 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. Several approaches have taken in an effort to increase the circulation time of liposomes by coating the liposomal surface with a hydrophilic polymer such as polyethylene glycol, but have new toxic effects appeared like skin toxicity generally known as “Hand-Foot Syndrome”. In the present study Doxorubicin Liposomes were formulated by “Double emulsion method to form Multivesicular liposomes”. The influence of various formulation and process parameters using different ratios of inner aqueous phase: oil phase: outer aqueous phase, homogenization speed, homogenization time on encapsulation efficiency, % free drug, particle size, zeta potential, surface morphology and release were investigated. Less than 10 % free drug was achieved with double emulsion method. The Scanning Electron Microscopy image showed the spherical shape having $33 \pm 5 \mu\text{m}$ particle sizes. The in-vitro drug release for optimized formulation was found to be controlled release of drug over a period of 7 days.

Key words: Liposomes, Doxorubicin HCl, lipids, double emulsion method.

INTRODUCTION

Doxorubicin hydrochloride is one of the most commonly used cytotoxic anthracycline antibiotics used in cancer chemotherapy and has been shown to have activity against a wide variety of neoplasms. The tumour must be exposed for a sufficient length of time so that all or most of the cancer cells have attempted to synthesize DNA in the presence of Doxorubicin¹. Conventional compositions of doxorubicin hydrochloride are 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. Severe myelosuppression, nausea, vomiting, alopecia, mucositis & cardio toxicity, limits the use of Doxorubicin HCl. It also causes extravasations & necrosis at the site of injection. Several approaches have taken in an effort to increase the circulation time of liposomes 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. The marketed preparation of Doxorubicin Hydrochloride in the form of Pegylated Liposomes is available on the name of DOXIL manufactured by Ben Venue Laboratories, Inc². 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”³. To overcome these problems, an alternative approach is needed. In the present study Doxorubicin Liposomes are Formulated by “Double emulsion method to form Multivesicular liposomes” to check effect of drug loading and particle size. The present investigation meets this need by providing compositions of multivesicular liposomes useful as a sustained release drug delivery system by intramuscular (I.M.) administration. Multivesicular liposomes contain multiple non - concentric aqueous chambers per particle within each liposome particle, resembling “foam” like matrix⁴.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride was purchased from Sterling Biotech, China. Di-Olelyl-Phosphatidyl Choline, Di-Palmitoyl-Phosphatidyl Glycerol, Triolein and Cholesterol were purchased from Lipoid Pvt. Ltd, Mumbai. The laboratory grade chemicals used for the work are Lysine HCl, Sucrose, Glucose, Sodium chloride, Chloroform, Sodium hydroxide, Triton, Acetonitrile are purchased from Merck Chemicals Pvt., Ltd. Mumbai.

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 interaction between the carriers and drug; The following IR spectroscopy was recorded.

Preparation of Doxorubicin HCl liposomes

Doxorubicin HCl Liposomes were prepared by double emulsion solvent evaporation method. Inner aqueous phase was prepared by dissolving Doxorubicin HCl in water and nitrogen was purged for 5 min after it got completely dissolved. Outer aqueous phase was prepared by dissolving glucose (5%) and lysine (100 mM) in W.F I and purged with nitrogen for 20 min. Lipid solution was prepared by dissolving weighed quantities of lipids, Di-Olelyl-Phosphatidyl Choline (1.425 gm), Di-Palmitoyl-Phosphatidyl Glycerol (0.250 gm), Triolein (0.300 gm) and Cholesterol (1.025 gm) in chloroform and dissolved the lipids by sonication for 5 min. Primary Emulsion (W_1/O) was prepared by adding drop by drop Inner aqueous phase into the lipid solution with 20 ml syringe by High shear homogenizer. Secondary Emulsion ($W_1/O/W_2$) was prepared by pouring the primary emulsion slowly in to the glass beaker containing the Outer aqueous phase by High shear homogenizer. The prepared secondary emulsion was transfer in to the round

bottom flask (RBF) containing aqueous solution 2 and rotated using Rota Vopour, to remove chloroform completely. After complete removal of chloroform, the solution present in RBF was transferred in to the centrifuge tubes, centrifugation was carried out for 20 min at 2000 rpm, supernatant was collected and sample was given for analysis of free drug. 100 ml of 0.9% NaCl was added to collected wet liposomes to remove the free drug and again centrifuged for 10 min at 2000 rpm. Wet liposomes were collected after removal of supernatant, added 5 ml of 0.9% NaCl and re-dispersed the wet microspheres by vortexing for 5 mins. It formed the red suspension of Doxorubicin liposomes and make-up with 0.9% NaCl if required^{4,5,6}.

Characterization of Liposomes

Percent free drug

Measure the absorbance of solution at 590nm using Glucose-Lysine solution as blank.

Transferred 0.1ml of sample to a 20 ml stoppered test tube, added 8ml of Glucose-Lysine solution to it, mix well, measure the absorbance 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 measure the absorbance 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 solution, mix well and measure the absorbance at 590 nm using calibrated UV (A₃)⁷.

Percent Free Doxorubicin Hcl = $[(A_2 \times 1.125) - A_1 / A_3 \times 1.25] \times 100$.

Doxorubicin HCl Assay

A standard and sample solution was prepared, injected separately 20 µl of the standard and sample solution in chromatographic condition and recorded the chromatogram. Calculate the content of drug per ml in liposomal injection as follows⁷.

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.

Particle size analysis and Zeta potential

The mean diameter and surface charge of liposomes was determined by laser diffractometer (Mastersizer X, Malvern Instrument, UK). Liposomes were diluted from 1 to 10 fold prior to determination of particle size and Zeta potential⁸.

Scanning Electron Microscopy (SEM)

The Morphology and surface appearance Liposomes were examined by using SEM (Using Hitachi-S-3700N). Scanning electron microscopy was carried out to study the morphological characteristics of Doxorubicin Hydrochloride Liposomes. The samples for the SEM analysis were prepared by placing the Liposomal solution on one side of adhesive stub. Then the dried liposomes were coated with gold (100A°) before microscopy. Finally the morphology of the Liposomes was observed with the scanning electron microscopy⁸.

In vitro Release studies

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 (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 λ max of 254nm^{9,10,11}.

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 40°C and room temperature for up to three months. The stability parameter, such as Assay was determined as function of the storage time.

TABLE 1: FORMULATION VARIABLES OF DOXORUBICIN LIPOSOME INJECTION

S. No	Formulation code	Ratios of W ₁ :O:W ₁	% Drug loading	% Drug release after 7 days
1	F1	1 : 1 : 2	83.54	85.43
2	F2	1 : 2 : 2	79.43	65.27
3	F3	1 : 2 : 4	92.63	84.13
4	F4	1 : 2 : 6	80.43	68.76
5	F5	1 : 4 : 4	79.64	54.53
6	F6	1 : 4 : 6	75.66	49.64

TABLE 2A: PROCESS VARIABLES OF DOXORUBICIN LIPOSOME INJECTION

Optimized Formulation (F ₃)	Primary homogenization speed (rpm)	Secondary homogenization speed (rpm)	Particle Size (µm)	Entrapment Efficiency (%)
F3 _A	12,000	5000	17	72.83
F3 _B	10,000	5000	34	92.52
F3 _C	8,000	5000	40	90.64
F3 _D	10,000	4,000	52	84.32
F3 _E	10,000	3,000	64	81.56
F3 _F	10,000	12,000	9	68.65

TABLE 2B: PROCESS VARIABLES OF DOXORUBICIN LIPOSOME INJECTION

Ideal Formulation (F ₃)	Primary Time (min)	Secondary Time (min)	% Entrapment Efficiency
F _{3B1}	5	1	91.42
F _{3B2}	8	1	86.45
F _{3B3}	1	1	36.62
F _{3B4}	5	4	85.64
F_{3B5}	5	1	92.38
F _{3B6}	5	6	40.83

TABLE 3: STABILITY DATA AT 25°C

Test	Initial	1 month	2 months	3 months
Description	Red colour suspension	Red colour suspension	Red colour suspension	Red colour suspension
% Free drug	8.62%	8.87%	8.98%	9.21%
Assay	101.0%	100.7%	99.7%	99.1%

Figure 1: FTIR of Doxorubicin hydrochloride

Figure 2: FTIR of doxorubicin hydrochloride, DOPC, DPPG, cholesterol, triolein

Figure 3: Particle Size distribution for F_{3B5} formulation

Results

	Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -18.9	Peak 1: -18.9	100.0	6.85
Zeta Deviation (mV): 6.85	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.200	Peak 3: 0.00	0.0	0.00
Result quality : Good

Figure 4: Zeta Potential for F3_{B5} formulation Figure 5: In-vitro release profile for F3_{B5} formulation**RESULTS AND DISCUSSIONS****Compatibility studies**

The compatibility between the drug, lipids 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 as shown in Figure 1 & 2.

Optimization of process and formulation variables for Doxorubicin liposomal

The Doxorubicin Liposomes were prepared by Double Emulsion technique and optimized the process-formulation variables such as ratio of inner aqueous phase (w_1) to organic phase (o) to outer aqueous phase (w_2) and homogenization speeds (primary and secondary) which was given in the Table 1 & 2. Out of six formulation variables, F3 was given highest % drug loading and more than 80% of drug was released after 7 days. Formulation F3 was selected as optimized compositions for further process variables parameters like primary and secondary homogenization speed shown in table 2 and primary and secondary homogenization time shown in table 3. There was a significant difference in particle size and entrapment efficiency on primary and secondary homogenization speed. Formulation F3B was selected as an optimized formulation as it was given maximum entrapment efficiency with desired particle size for further optimization

parameters. There was a significant difference in particle size on primary homogenization time but no effect was observed of secondary homogenization time on particle size. Formulation F3_{B5} was chosen for further characterization of doxorubicin liposomes^{12,13,14,15}

Percent free drug

The percent free drug was determined for optimized formulation F3_{B5}. The percent free drug for F3_{B5} formulation was obtained 8.62%.

Doxorubicin HCl Assay

The Assay was determined for the optimized formulation F3_{B5}. The assay for F3_{B5} formulation was achieved 101% which contained 8.62% of free drug and 92.38% of entrapped drug.

Particle size analysis and Zeta potential

The particle size distribution was analyzed for optimized formulation F3_{B5} of Doxorubicin HCl Liposomes by wet method. The particle size and Zeta Potential for F3_{B5} was 33.39 μ m (Figure 3) & -18.9mV (Figure 4) respectively.

Scanning Electron Microscopy (SEM)

The Morphology and surface appearance of Liposomes were examined by using SEM (Figure 5). The SEM image for F3_{B5}

formulation showed that the liposomes have spherical shape having $33 \pm 5 \mu\text{m}$ particle sizes.

In vitro Release studies

The in vitro dissolution profile for F3_{B5} formulations (Figure: 6) was carried out by membrane diffusion method. The dissolution was carried out for a period of 7 days in saline phosphate buffer pH 7.4. Initial burst release (1day) of liposomes was 26% and more than 90% of drug was release within 7 days.

Stability Studies

The stability of the Doxorubicin liposomes was evaluated for optimized formulation of F3_{B5} after storage at accelerated condition at $25 \pm 2^\circ\text{C}/60 \pm 5\%$ RH for 3 months. The Description, % free drug & assays of the samples were determined as a function of the storage time. The Liposomes stored at 25°C were found to be stable for duration of 3 months. The results were showed in Table 3.

CONCLUSION

In the present study, attempts were made to prepare Doxorubicin HCl Liposomes for controlled release by double emulsion solvent evaporation technique. The selection of different ratios of Inner aqueous phase: Oil phase: Outer aqueous phase, speed of primary homogenization and time of primary and secondary homogenization were found to have played a predominant role in the preparation. The prepared liposomes were evaluated for % free drug, % assay, particle size, and zeta potential. More than 90% drug was entrapped with double emulsion method. The optimized formulation was exhibited more than 90% release of drug for a period of 7 days. From the experimental results it was evidenced that the controlled release of Doxorubicin liposomes was successfully formulated with less side effects.

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