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RESEARCH ARTICLE

Formulation Development and evaluation of Liposomal Drug Delivery System Containing Etoposide

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ABSTRACT

Two most commonly used preparative methods, reverse phase evaporation and ethanol injection were employed to prepare cationic liposomes composed of Etoposide API, DMPG-Na polymer and Cholesterol binder, respectively. To overcome the hindrances of the reported HPLC analytical method in pharmacopeia which requires more time in preparation for solvent and also its bit tedious; we have developed and validated a simple method which will be applicable to detect and quantify actual drug in formulation as well as it can be applied for pharmacokinetics study. The resulting formulations were evaluated through morphology observation, particle size and zeta potential analysis, % entrapment efficiency and % drug loading. The results showed that liposomes prepared by ethanol injection method were of best quality and stability, with promising results. However ETNLE 5 shows best results i.e. particle size 197.3±0.21nm, polydispersity index 0.340±0.051%, and zeta potential of about -12.7±1.266mV. Entrapment efficiency 81.78± 0.78% and drug loading 89.62±2.53% is the highest as compared to all other batches. % In-vitro Drug release study showed 15% and 21% of drug was released in the first five minutes with a cumulative drug release of 58% and 78% for ETNLE 5 formulation at pH 1.2 and pH 6.4 respectively. Stability study of optimized batch showed no significant changes in evaluation parameters. Cell viability study on A-549 cells by MTT assay clarified cancer cells are inhibited by 200 µM equivalent etoposide liposomes as compared to 64.88% of free drug. These findings clarified the effect of preparative methods on performance of cationic liposome, as well as formulation factors on entrapment efficiency, and will provide important methodological reference for further study of liposomes carriers for drug delivery to tumor penetration.

KEYWORDS:
Etoposide; Liposome; Entrapment efficiency; Ethanol injection; Reverse phase evaporation.

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INTRODUCTION

The death rate due to cancer is still growing worldwide [1, 2]. Thus, the research related to the disorder and its treatment is continuously at spotlight. The different clinical strategies like therapies and drug delivery systems have been restructure and rediscovered on regular and at rapid rate. The combinational therapies need to be paid crucial observations as the drugs have very small therapeutic index [3]. There are various challenges like pharmacokinetics, in-vivo distribution (especially when drugs are given in combination), the specificity of tumor target, accumulation of drug. Even the high number of side effects hampering the health more during the treatments [4].

Nano-based drug delivery system (NDDS) in this case has been proved to be essentially efficient choice line of treatment. It has ability to overcome many of the pharmaceutical and clinical hurdles which are faced during the chemotherapy. NDDS exhibits improved drugs stability, desired Pharmacokinetics patterns, targeted delivery system [5, 6, 7]. It also increases possibility of combination of different type of drugs in a single formulation, thus holds a big share in developing effective chemoimmunotherapy regimens.

Liposome is one of the most widely explored area in NDDS [5]. It has huge applicability by inducing different manipulations in the structure as well as the excipients used. The spectrum of variations in this delivery system that are

possibly makes it more viable formulations for many drugs as well as treatments. The other reason for its acceptability is the structure of liposomes. As, it can carries both hydrophilic and lipophilic type of drugs in one closed vesicle [8]. This is possible due to its two-layered structure with phospholipids and cholesterol [9]. This combination facilitates higher encapsulation, targeting ability, low toxicity and primarily its feasibility to produce at industrial level.

Liposomes possess advantage over others are enhanced solubility of drug which is encapsulated, prevent the drug from biological and chemical degradation, decrease the toxic effect of drug, enhance therapeutic index and efficiency of drug entities, compatibility of drug increases with non-toxic and biodegradable materials [10]. The separation of inner core from outer phase improves the stability of the drug enclosed inside the formulation. Encapsulation of drug leads to gain controlled or sustain release of drug. The bioavailability of poorly soluble drugs can be improved using this technique. The change in the biological characters can be achieved by modifying the ligands on the surface of liposomes [11].

There are various methods for formulating liposomes. The selection of the technique is based on the following factors: 1) the physicochemical properties of the components of liposome and the drug to be loaded; 2) amount and toxicity of drug to be loaded; 3) form of medium which is used to dispersed the liposomes; 4) the additional processes during the application/delivery of the liposomes; 5) the size and the half-life desired for the successful application; and 6) the costs, reproducibility, and applicability regarding large-scale production for clinical purpose and good manufacturing practice-relevant issues[10, 11].

The methods employed to formulate liposomes includes supercritical fluid technology, dual asymmetric centrifugation, membrane contactor technology, cross-flow filtration technology and freeze drying technology. The more commonly used method to prepare liposome is Bangham or thin film hydration method [12, 13]. The method involved the solubilization of lipid in organic solvent, evaporation of the solvent and dispersion of the lipid film in aqueous medium. The techniques such as solvent injection and reverse- phase evaporation provide hydration of lipids from organic solvent and produce unilamellar vesicles (ULVs) and multilamellar vesicle (MLVs) [14, 15]. The advantage of solvent injection and reverse- phase evaporation method is to produce high encapsulation efficiency than thin film hydration technique. It has been documented by many researchers that liposomes formulated with these two methods are very stable and also give higher encapsulated yield.

Thus, for a drug like etoposide which is an anti cancer agent mainly targeting the small lung cell tumour, liposome is good choice of delivery system.

Etoposide is an antineoplastic agent, derived from epipodophyllotoxins. Chemical form of etoposide is [(4'-demethyl-epipodophylotoxin 9-[4,6-O-(R)-ethylideneß-D-glucopyranoside]. The drug is useful in the treatment of lung cancer, testicular cancer and lymphomas[16, 17]. The action of topoisomerase II which is involved into DNA uncoiling is inhibited by the drug which ultimately effects on its repairing and replication [18]. The drug also administered with other anticancer drugs produce different actions [19]. Low solubility of the drug in water, leads to insufficient drug transport to the target lesion. Oral administration of drug leads to poor bioavailability (57%) [20].

The present work involved the preparation of nanoliposomes by reverse phase technique using Rota-vapor and ethanol injection method. We aim to formulate rapid and stable liposomes so that we can deliver the drug in oral form with higher bioavailability. This can be achieved as we reduce the size of liposome with higher entrapment efficiency. Based on particle size and entrapment efficiency the best method and the formulation has been selected for further detailed study.

Experimental work

Chemical and material

The drug Etoposide was procured as gift sample from Naprod Life Sciences Pvt. Ltd. Mumbai, Methanol of HPLC grade was purchased from Merck.

The chemicals required to prepare liposome such as DMPG-Na was procured as gift sample from Lipoid, Germany and ethanol and chloroform were purchased from LOBA Chemie Pvt. Ltd., Mumbai.

METHODOLOGY

Preformulation Study

The standardization of drug and drug-excipients interaction was carried out based on the various parameters including physicochemical properties of drug, drug interaction by DSC and FTIR studies.

Fourier Transform Infrared Spectroscopy [21]

Infrared spectra were generated with an FTIR spectrophotometer.The spectra were obtained using KBr pellet technique. The KBr pellets were prepared by 10 mg sample mixed with 200 mg potassium bromide at high compaction pressure. Thus, the prepared pellets were scanned at a resolution of 4000 cm -1 to 400 cm -1.

Differential Scanning Calorimetry

DSC thermal studies was carried out to observe the thermal behavior of the Etoposide and excipients on DSC 25 Mettler (Perkin-Elmer). The samples were heated in sealed aluminum pans which were heated at a rate of 50 °C/min over a temperature range from 50 ± 1°C to 300°C. Nitrogen gas purged at a rate of 30 ml/min. Empty aluminum pan was used as a reference [22].

Method for preparation of liposome

Ethanol- injection Method

The formulations with different combination of excipients given in Table 1 were prepared by mixing amount of Etoposide with phospholipid (DMPG-Na) and amount of cholesterol in the ethanol and chloroform mixture (1:1). The solution was sonicated for 15 min using ultrasonic water bath. The prepared solution was then quickly added in 25ml of deionized water and kept on magnetic stirrer at 500 rpm using syringe. Continue the stirring until evaporation of chloroform and ethanol is completed. After 6-8 hrs of

stirring, the nanoliposomal suspension were obtained. Finally, demineralized water was added to adjust the volume up to 25ml. Stabilized the formed liposome by keeping in the refrigerator for at least 6 hrs.

The composition of the formulations ETNLE1, ETNLE2, ETNLE3, ETNLE4, ETNLE5, ETNLE6, ETNLE7 by ethanol injection method are given in Table 2.

Table 1: Drug to polymer Ratio in Nano-liposome (NLs) Formulations by Ethanol Injection Method

Sr. No.	Formulation Code (ETNLE and ETNLRP)	Ratio		
		Drug : polymer (DPMG-Na)	Drug: Cholesterol	Ethanol and Chloroform
1.	1	1:2	1:1	1:1
2.	2	1:3	1:1.5	1:1
3.	3	1:4	1:2	1:1
4.	4	1:5	1:2.5	1:1
5.	5	1:6	1:3	1:1
6.	6	1:7	1:3.5	1:1
7.	7	1:8	1:4	1:1

Table 2: Composition of the Etoposide Nano-liposome (NLs) Formulations by Ethanol Injection Method

Sr. No.	Name of Ingredients	ETNLE 1	ETNLE 2	ETNLE 3	ETNLE 4	ETNLE 5	ETNLE 6	ETNLE 7
1.	Etoposide	5 mg	5 mg	5 mg	5 mg	5 mg	5 mg	5 mg
2.	DMPG- Na lipoid	10 mg	15 mg	20 mg	25 mg	30 mg	35 mg	40 mg
3.	cholesterol	5 mg	7.5 mg	10 mg	12.5 mg	15 mg	17.5 mg	20 mg
4.	Ethanol and Chloroform (1:1)	2.5 ml 2.5 ml	3.75 ml 3.75 ml	5 ml 5 ml	6.25 ml 6.25 ml	7.5 ml 7.5 ml	8.75 ml 8.75 ml	10 ml 10 ml
5.	Deionised water	25 ml	25 ml	25 ml	25 ml	25 ml	25 ml	25 ml

Reverse phase technique using Rota-vapor

The Etoposide (API), DMPG-Na and cholesterol were added to a 100 ml round-bottom flask with a long extension neck containing solvent mixture comprising of ethanol and chloroform (1:1). The round flask was rotated at a constant rpm of 150 dipped in a temperature controlled water bath maintained at 40°C using a Rotary Evaporator. Solvent was removed under reduced pressure by using a vacuum pump [23, 24]. A thin film was formed after complete evaporation of solvent mixture. The lipids were redissolved in the organic phase and increase the solubility of lipids using chloroform. The aqueous phase was prepared by dissolving Mannitol in 25ml of deionized water with the aid of heat. The prepared aqueous phase was added to the above prepared organic phase. Sonicated the mixture for 15 mins with pulse rate of 3 seconds followed by 1 second interval (no pulse) and at amplitude of 80% in a bath-type (in cold condition). The mixture was sonicated until a homogeneous opalescent dispersion was obtained. The mixture was placed on the rotary evaporator to remove the organic solvent under reduced pressure {vacuum} at 20-25°C, rotating at approximately 180 rpm.A viscous gel was formed exhibiting an aqueous suspension. An excess deionized water was added to the formed suspension and evaporated for an additional 15 min at 20 °C to remove the traces of solvent. Liposomes formed were observed under an oil emulsion microscope (100X).

The composition of the formulations ETNLRP1, ETNLRP2, ETNLRP3, ETNLRP4, ETNLRP5, ETNLRP6, ETNLRP7 by reverse phase technique are given in Table 3.

Table 3: Composition of the Etoposide Nano-liposome (NLs) Formulations by Reverse phase technique

Sr. No.	Name of Ingredients	ETNLRP 1	ETNLRP 2	ETNLRP 3	ETNLRP 4	ETNLRP 5	ETNLRP 6	ETNLRP 7
1.	Etoposide	5 mg	5 mg	5 mg	5 mg	5 mg	5 mg	5 mg
2.	DMPG- Na lipoid	10 mg	15 mg	20 mg	25 mg	30 mg	35 mg	40 mg
3.	Cholesterol	5 mg	7.5 mg	10 mg	12.5 mg	15 mg	17.5mg	20 mg
4.	Ethanol and Chloroform (1:1)	2.5 ml 2.5 ml	3.75 ml 3.75 ml	5 ml 5 ml	6.25 ml 6.25 ml	7.5 ml 7.5 ml	8.75 ml 8.75 ml	10 ml 10 ml
5.	Deionised water	25 ml	25 ml	25 ml	25 ml	25 ml	25 ml	25 ml

Development and validation of selected formulation of Etoposide using HPLC as per ICH guidelines

The reported HPLC analytical method in pharmacopeia requires more time in preparation for solvent and also its bit tedious. We aimed to develop a simple method which will be applicable for the analysis of our formulations as well as it can be applied for pharmacokinetics study.

The estimation of the drug was achieved by reverse-phase high performance liquid chromatography. The determination was done using Agilent Tech. (1100) system. The separation of chromatogram was carried out on column Fortis C18 (100 x 4.6 mm id with 2.5µm particle size) using acetonitrile water and water with 0.15% of OPA (Ortho phosphoric acid) as a mobile phase. The detection was done at 283 nm. The flow rate was maintained at 1ml/min. Quantification of drug was estimated by calculating peak are using CHEMSTATION 10.1 software.

Preparation of mobile phase

Mobile phase was prepared by mixing HPLC grade distilled water, acetonitrile, and distilled water with 0.15% Ortho phosphoric acid (OPA) in the ratio of 50:30:20 v/v. The pH of solvent system was maintained at 1.5. The content was sonicated for 15 min and filtered through 0.45 µm membrane filter. Mixed solvents were degassed and used as mobile phase.

Standard preparation

Standard stock solution of etoposide with concentration of 1000µg/ml was prepared by dissolving API in methanol.

The further dilutions were made with the concentrations from 10-1000 µg/ml by diluting amount of standard stock solution in the mobile phase. Fresh solutions were prepared daily, filtered, and degassed by sonication.

Determination of wavelength

It is the characteristic of compound which helps to provide the electronic structure of the compound or analyte. The spectral analysis under UV ranging from 200- 400nm was carried out by using standard solution of the Etoposide.

HPLC method Development

The optimization of the RP-HPLC chromatographic parameters were carried out by using different compositions of mobile phase. The resolution and peak symmetry obtained with a mobile phase distilled water, acetonitrile, and distilled water with 0.15% Ortho phosphoric acid (OPA) in the ratio of 50:30:20 v/v at pH 1.5 was excellent. Based on peak area quantification was carried out at 283nm. System suitability was evaluated for the proposed method. The system suitability test was carried out on freshly prepared standard stock solution of Etoposide. Parameters such as resolution, peak tailing, HETP were studied to evaluate the suitability of the system.

Method validation parameters

1. Linearity

The calibration curve was plotted over the concentration range from 20 to 100 µg/ml of Etoposide. The aliquots of each solution were injected under the optimized chromatographic conditions. The regression equation and correlation coefficient were evaluated by plotting peak area versus concentration of Etoposide.

2. Accuracy

The accuracy of method was evaluated by the standard addition method. The known amounts of standard solutions such as 80, 100, 120% levels were added to previously analyzed sample solutions of drug. The percent of individual recovery and % Relative standard deviation (RSD) at each level for the drug was measured. The solutions were analyzed triplicate in each level.

3. Precision

Method precision (repeatability)

The repeatability of instrument was tested by repeatedly injecting solution of drug of concentration 40 µg/ml. The % RSD should not be more than 2%.

Intermediate precision

Intermediate precision was evaluated through intraday and interday precision. The intraday precision was examined using three different concentrations. The intraday and interday precision was studied by analyzing the corresponding concentration 3 times on the same day and on different days. The results were described in terms of % relative standard deviation (% RSD).

4. Robustness

The robustness of method was estimated by changing the optimized conditions. In present study the method was examined by deliberate alterations in the chromatographic conditions such as detection wavelength (± 1 nm), flow rate (± 0.1 ml/min) and mobile phase. The % RSD was determined.

5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) of the method were determined by visual method on the basis of trial and error. Calculation of LOD and LOQ were carried out from signal-to-noise ratio for all three API. S/N ratio was determined by spotting each drug at various concentrations until S/N ratio of LOD was 3 and of LOQ 10.

Optimization of formulation

The selection of optimized formulation was done based on the following parameters particle size, poly-dispersibility, zeta potential, percent entrapment efficiency, drug loading TEM.

Particle size and poly-dispersibility

The measurement of particle size and poly-dispersibility were done on Zetasizer instrument (Malvern, serial no.: MAL1074174) at 25°C. The analysis was done by the software provided by Malvern Instruments. Before analysis samples were placed in refrigerator maintained at 4°C.

Zeta potential

The formulation of liposome was tested for zeta potential using Malvern Zetasizer instrument. The analysis was carried out at 25°C.

% Entrapment Efficiency and Drug Loading [25]

The entrapment efficiency and drug loading of nano-liposomes (NLs) was determined by separating non-encapsulated etoposide from etoposide NLs suspension by centrifugation 2 mL of ETNLs at 20000 RPM for 15 min at 4°C.

The supernatant was discarded and the sediment nano-liposomes were disrupted with 2 ml ethanol to release the entrapped drug; suitably diluted with water up to 10 ml and analyzed for drug content at 283 nm to calculate the entrapment efficiency.

$$\text{EE} = \frac{\text{Total quantity of Etoposide} - \text{Concentration of Etoposide in the liquid}}{\text{Total quantity of Etoposide}} \times 100$$

$$\text{Loading efficiency (\%)} = \frac{\text{Amount of drug in nanoparticle (mg)}}{\text{Amount of nanoparticle (mg)}} \times 100$$

Drug loading was determined using HPLC. 1 ml of nanocochleate formulation was dissolved in 1 ml ethanol and EDTA (1:1) made the volume upto 10 ml using deionized water. The solution was then sonicated for 5 min. The resulting solution is then filtered using 0.45 µm filters. The filtrate was then analyzed through HPLC.

Evaluation of API and optimized nano-liposomal formulation

1. In-vitro Drug Release:

In-vitro release of drug from raw materials and nanoparticles [26-28] was carried out by the dialysis membrane method. The amount of formulation equivalents to 50 mg of drug was taken in dialysis bags (with a cutoff of 12,000 Da, Sigma). The drug loaded dialysis bag suspended in a beaker with 100 ml of phosphate buffer saline maintained at pH 1.2 was kept on a magnetic stirrer which is rotated at 100 rpm, with temperature adjusted to 37±0.5°C for a selected time interval. Withdraw a sample of 5 ml for analysis and replaced the same quantity with a fresh buffer solution. The samples was then filtered through 0.45µm filter. The samples were analyzed for drug release by determining absorbance at 284 using UV - Visible spectrophotometer, the rate of etoposide release obtained using the standard curve. Followed the same procedure for phosphate buffer saline maintained at pH 6.8.

2. Stability Stability:

Freshly prepared optimized drug-loaded nanoparticles suspension was transferred into a glass vial and stored at 4°C for 1 year for stability study. Samples were regularly withdrawn to perform size measurement, EE and zeta potential have been checked after stability study. [27]

3. MTT assay [29]

The prepared nano-formulations of Etoposide were evaluated for the efficacy against cancer cells. The cell employed was human cells for lung cancer A549 cells. MTT assay was used to determine quantitative cytotoxic activity of Etoposide.

Cell line and Reagents and kits

A549 lung cell line was obtained by National centre for cell science (NCCS) Pune, India. Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate buffer saline were purchased from Invitrogen (Carlsbad, USA). All other chemicals and reagents used in this study were of analytical grade.

Cell culture and culture conditions

A549, adenocarcinomic human alveolar basal epithelial cell line was obtained from National Centre for Cell Science (NCCS, Pune, India). A549 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin and 50 units/ml penicillin. The cells were incubated at 37°C in the presence of 5% CO2 and were sub-cultured every 2 days.

Cytotoxicity assay

About 70- 80% confluent cell lines were collected and checked for viability. Number of cells was determined using hemocytometer. Cell suspension was centrifuged, and pellet obtained was suitably diluted with medium. A 96 well plate was seeded with 5x105 cells/well and incubated for 24 hrs at 37°C, 5 % CO2.Dilutions of test formulations as well as pure drug solution were prepared with concentration of 20-200µM. Placebo formulations of all the formulations also were subjected to dilution in the same way as that of drug containing formulation. The dilutions were prepared using media as vehicle. The plate was labelled and to each well 100 µl of test solution was added. The experiment was performed in triplicate for each concentration. Plate was again incubated for 24 hrs at 37°C, 5 % CO2. Untreated cells were considered as control. After 24 hrs incubation media was removed from each well and the wells were washed with buffer to remove traces of formulation and fresh media and MTT solution (20 µl) was added in each well. Plate was incubated for 4 hrs at 37°C in presence of 5 % CO2. After incubation, the MTT reagent was discarded and DMSO was added to solubilize the formazan crystals and absorbance was determined at 570 nm. vii. The % inhibition was calculated using following formula:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100$$

From % inhibition the % of viable cells was calculated and plotted on Y-axis against the logarithm of concentration (mcg/ml) (log10 conc) on X- axis. IC50 value was determined from the graph and linearity equation.

4. Surface morphology by TEM

The optimized etoposide-NL was studied for surface morphology using transmission electron microscopy (TEM).

5. Pharmacokinetic study

After the approval from the Institutional Animal Ethics Committee (Protocol No. IIP/IAEC/06/2019-20). Albino Wistar rats of 200-250 g weight were used for pharmacokinetic study. The animals were kept fasted overnight with free access to water throughout the experimental period. The 9 mg/kg dose of API and optimized formulations (nanoliposomes and nano-cochleates) were administered to the Wister male rats (n= 10) except normal group by oral route. Collected the 0.25 ml of blood samples from the retro-orbital plexus mild anesthesia with isoflurane in micro-centrifuge tubes containing 10 µl of EDTA at the predetermined time (from 15-240 min). The plasma was collected from the samples by centrifugation process at 4000 rpm for 10 min. The obtained plasma samples (200 µl) were stored at -20 °C till the examination of drug concentration. The aliquot was then used for HPLC analysis. The pharmacokinetic parameters were evaluated by a non-compartmental model with the aid of the add-in program PKSolver [30]. All values were expressed as their mean ± SD. Differences were regarded to be significant as the level of $p < 0.05$.

6. Tissue bio-distribution study

To investigate the bio-distribution of free API and nanoliposomes, 12 male Wistar rats (200 g) were arbitrarily divided into two groups (six in each). Free API and nanoliposome were administered by oral route at dose 9 mg/kg API equivalent respectively. Rats were sacrificed by excess anesthesia after 2 h following administration of the dose. The liver, lungs, brain, spleen, kidney, and heart were harvested and weighed. Two hundred milligram of tissue was homogenized with methanol (5 mL), centrifuged at 20,000 rpm and 4°C for 20 min. Later, the filtered supernatant was introduced into the HPLC system to determine API concentration [31].

RESULTS AND DISCUSSION

Preformulation Study

The results for identification tests as per monograph are given in Table 4

Table 4: Physicochemical Properties

Sr. No.	Test	Observation	Inference
1.	Description- Solid white powder	Solid white powder	Complies
2.	Solubility- Very soluble in methanol, chloroform; slightly soluble in ethanol, sparingly soluble in water	Very soluble in methanol, chloroform; slightly soluble in ethanol, sparingly soluble in water	Complies

Drug Excipient Compatibility study

Fourier Transform Infra-red spectroscopy (FT-IR)

Compatibility of drug with phospholipid and cholesterol were studied by FTIR spectroscopy using KBr pellet technique. The FTIR spectrum was shown in Figure 1. An interpretation of FTIR spectrum was shown in table 5

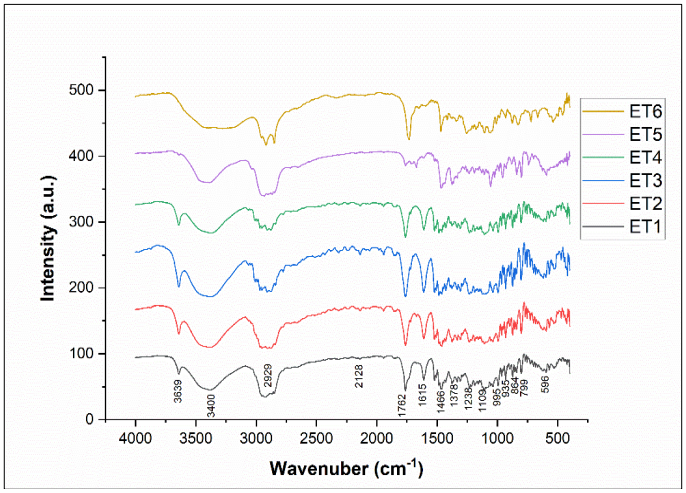


Fig.1: FTIR Spectra of Drugs and Excipients

(ET1: Etoposide with cholesterol and DMPG- Na; ET2: Etoposide with Cholesterol; ET3: Etoposide with DMPG-Na; ET4 Etoposide; ET5: Cholesterol; ET6: DMPG- Na)

Table 5: Major peak observed in FTIR spectra of Etoposide with Phospholipid and Cholesterol

Sr. No.	IR Range (Cm-1)	Functional group	Peak observed (Yes/No)		
			Etoposide	Etoposide and Phospholipid	Etoposide and Cholesterol
1.	3400-3000	OH	Yes	Yes	Yes
2.	1750-1650	C=O	Yes	Yes	Yes
3.	1190-1050	C-O	Yes	Yes	Yes
4.	750-650	C-H	Yes	Yes	Yes

From the above interpretation data, the results shown that there is no appearance of chemical interaction between drug and polymers in prepared nanoparticles and it confirmed the occurrence of drug as molecular dispersion in the polymeric nanoparticles.

Differential Scanning Calorimetry

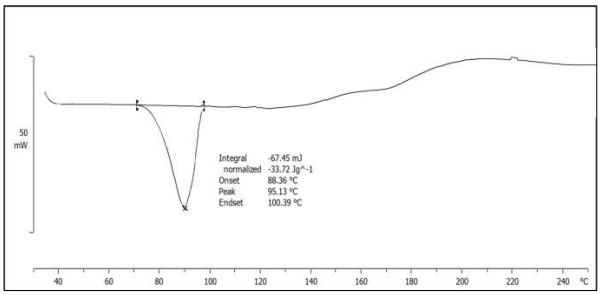


Fig.2: DSC thermogram of Etoposide

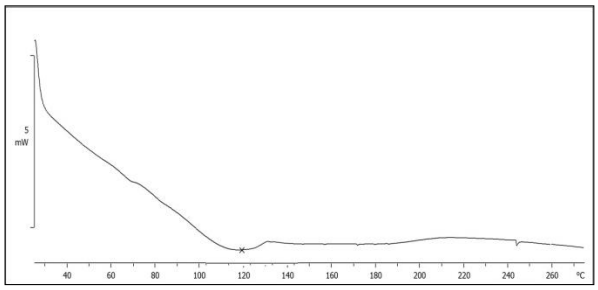


Fig.3: DSC thermogram of DMPG- Na

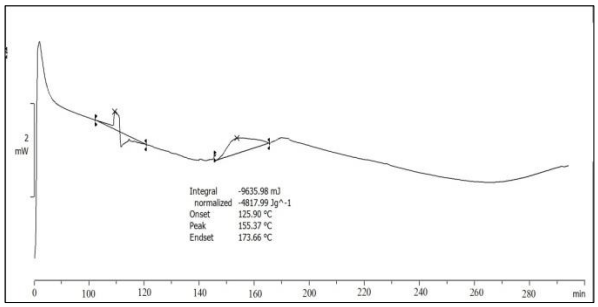


Fig.4: DSC thermogram of Cholesterol

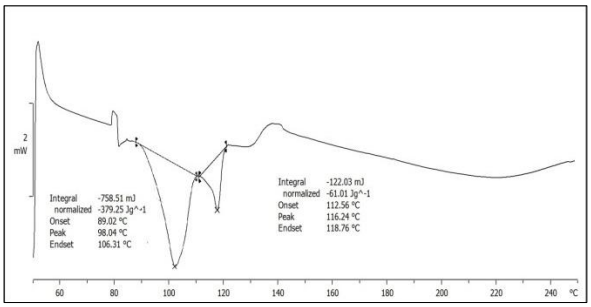


Fig.5: DSC thermogram of Etoposide Phospholipid

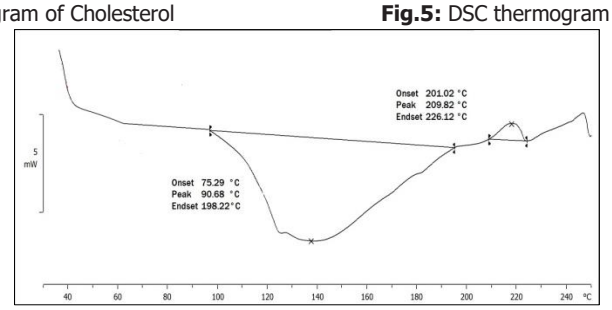


Fig.6: DSC thermogram of Etoposide Cholesterol

The DSC findings on Etoposide, DMPG, Cholesterol and binary mixtures are shown above in figure 2, 3, 4, 5 and 6. TheDSC curve of Etoposide (Figure 2) showed one sharp endothermic event at 95.13 °C (T onset = 88.36 °C) indicating the melting. The DSC curve of DMPG (Figure 3) has no significant events. The DSC curve of cholesterol (Figure 4) has no significant events. One exothermic event at 155.37 °C (T onset = 125.90 °C). The DSC curve for mixture of Etoposide and DMPG (Figure 5) showed two events. First at around 98.04 °C (T onset around 89.02 °C) an endothermic event which corresponds to the Etoposide and second endothermic event at 116.24 °C (T onset = 112.56 °C) an endothermic event which is also corresponds to DMPG which could be uncaptured due to broad nature however is peak could be seen around 118.00 °C. The DSC curve for mixture of Etoposide and Cholesterol (Figure 6) showed two events. First at around 90.68 °C (T onset around 75.29 °C) an endothermic event which corresponds to the Etoposide and second exothermic event at 226.12 °C (T onset = 209.82 °C corresponds to cholesterol. The endothermic events in both the mixture at melting point of Etoposide are superimposable and indicates no interaction with the excipient or no change in the physicochemical properties of the drug.

Development and validation of Etoposide using HPLC as per ICH guidelines

Selection of wavelength maxima

The solution of drug was scanned between ranges 200-

400nm. UV spectra of the drug show maximum absorbance at 283nm (Figure7).

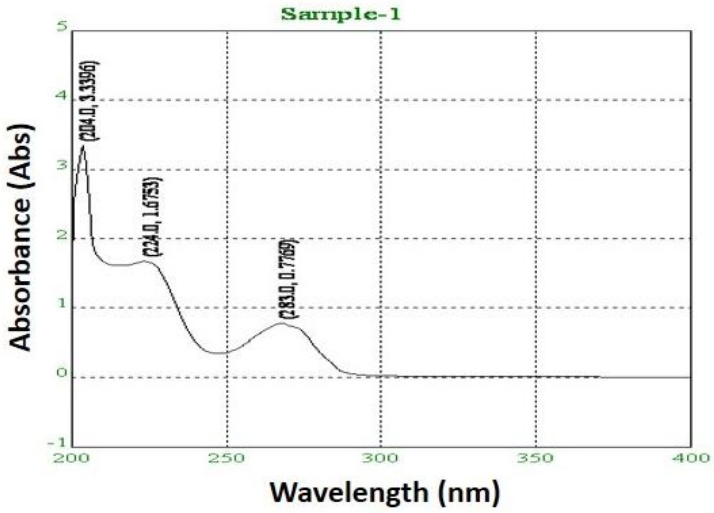


Fig.7: UV spectra of Etoposide between 200 – 400 nm

Optimization of RP- HPLC method

Good resolution, peak shape, theoretical plates, retention time and asymmetry are the main parameters which determine the optimization of any chromatographic method for analysis. Thus, to attain all these parameters, several conditions of chromatographic techniques were optimized and examined for the assessment of Etoposide, such as different compositions of mobile phase, flow rate, various stationary phases. The obtained peak was observed to be good, sharp, symmetrical, well resolved with mobile phase Water: Acetonitrile: water (0.15% of OPA) in a ratio 50:30: 20 v/v), with flow rate of 1 mL/minute at the analytical wavelength 283 nm. The retention time of Etoposide was observed at 10.13 min (Figure 8).Optimized characteristics are given in table 6.

Table 6: Optimized Characteristics

Sr. No.	Parameters	
1.	Composition of Mobile phase	Water: Acetonitrile: water (0.15% of OPA) in a ratio 50:30: 20 v/v
2.	Column Specifications	FortisC18 (100 x 4.6 mm id with 2.5 µm)
3.	Flow Rate	1.0 ml/min
4.	Retention Time	10.13 min
5.	Symmetry	0.89

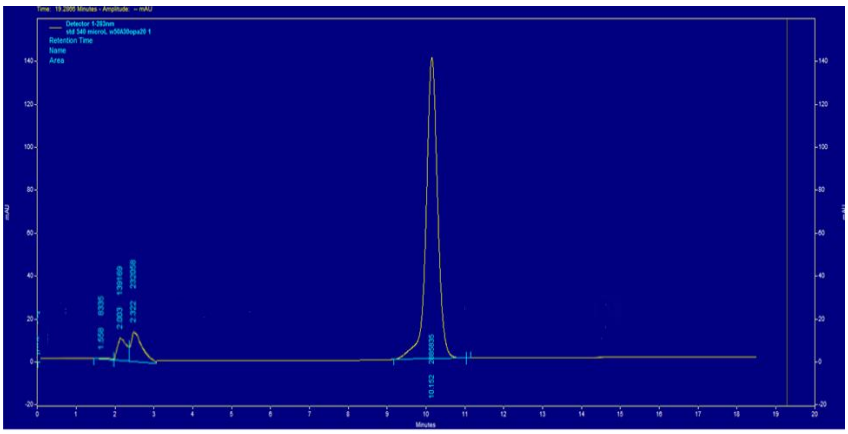


Fig.8: Chromatogram of Etoposide

Method validation parameters

1. Linearity

The linearity of the method was determined by diluting the standard stock solution to produce the concentration ranges from 5 to 25 µg/ml. The results show excellent correlation existed between peak area and concentration of analyte. By plotting the AUC versus the concentration of analyte, the calibration curve was prepared and analyzed through linear regression (Figure 9).

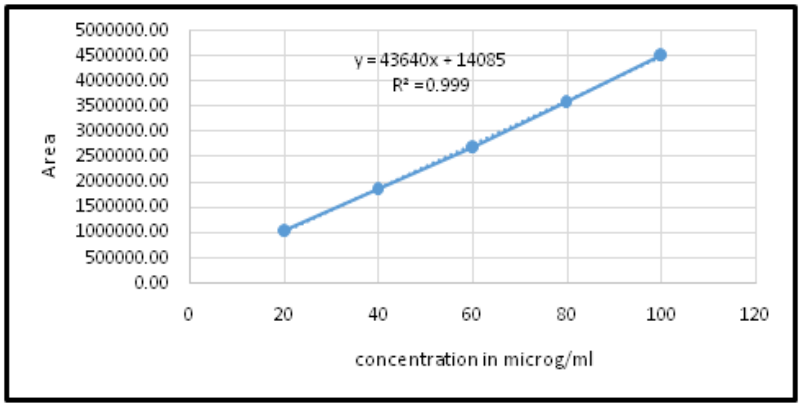


Fig.9: Linearity curve of Etoposide

2. Accuracy

Good recovery study of the drug was carried out at three different concentrations levels indicating the method was accurate. A known amount of standard drug (80, 100, 120%) was added into pre-analyzed sample and subjected them to the proposed HPLC method. The % recovery values for the API at three different level (80%, 100% and 120%) were 99.42%, 98.09% and 101.58% found to be within the limits.

3. Precision

Method precision (repeatability)

Method precision was evaluated by repeatedly introducing 60µg/ml concentration of etoposide. The developed method was found to be precise as % RSD was found to be 0.46.

Intermediate precision

The analysis of three different concentration (40, 60, 80 µg/ml) of standard solution showed good reproducibility. The % RSD was found to be 1.49, 1.08 and 1.28 for interday precision and 1.44, 1.04, and 0.96 for intraday precision.

4. Robustness

Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate and wavelength. The % RSD obtained by changing flow rate, wavelength, and mobile phase were 0.92% and 1.13%, 1.33% and 0.75%, 0.94% and 1.77% respectively. It was observed that there were no marked changes in the chromatograms. The developed method was found to be robust as the % RSD values were< 2.0 %.

5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

This data showed that the sensitivity of method to determine the etoposide. The LOD and LOQ was found to be 0.3924µg/ml and 1.1887µg/ml respectively.

Optimization of formulations

1. Particle size analysis, poly-dispersibility indices and Zeta potential measurement

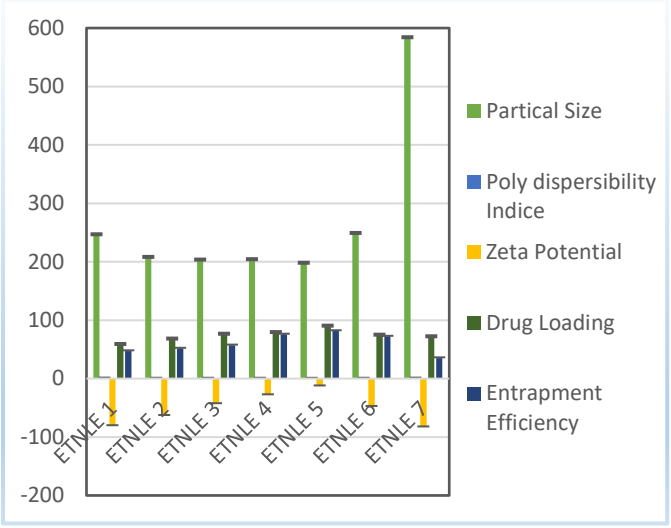
The particle size of nano-liposomes containing etoposide were determined using Malvern zeta sizer (DLS, Malvern instruments Inc., Malvern, UK). The polydispersity index (PDI) which measures the width of the size distributions was used to monitor the quality of the data. The particle size of the nano-liposomal formulation was ranges between 197.3±0.21 nm to 583.3±7.39 nm Table 7. The formulation ETNLE 5 showed lowest particle size that is 197.3±0.21 nm. The polydispersity indices were range between 0.340±0.051 - 0.833±0.006 which indicate that the liposomes were monodispersed to polydispersed. The zeta potential was found to be in the ranges between -12.7±1.266 mv and -82.9±0.513 mv (Table 7). The formulation ETNLE 5 showed better zeta potential given in figure 10.

The surface charge of the nano particle determines the zeta potential which is a paramount factor for the stability of nano formulations. This is turn is majorly responsible of the primary absorption of drug (which is in nano delivery system) onto the cell membrane. Once absorption is done, the particle size and polydispersity index play the vital role in the endocytotic uptake rate. Thus, particle size and zeta potential are the key factor for drug penetration into cells and tissues and nanoparticle toxicity [32, 33].

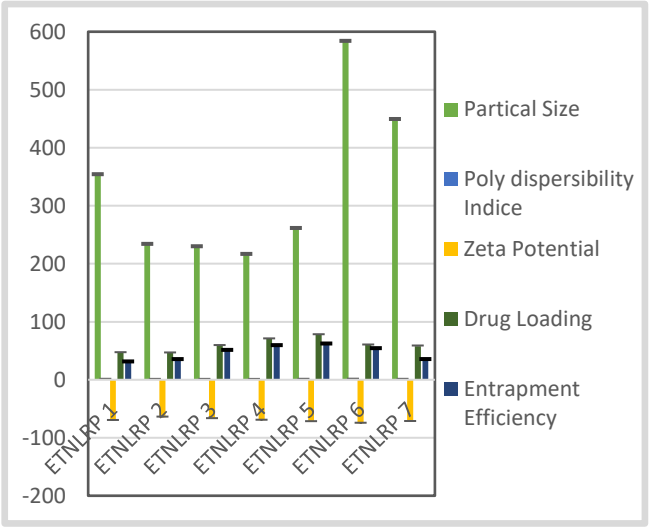
Table 7: Evaluation of Formulations

Sr. No.	Formulation	Particle Size (nm)	Poly-dispersibility	Zeta Potential
1.	ETNLE 1	246.0±0.26	0.718±0.001	-80.8±0.321
2.	ETNLE 2	207.3±0.95	0.453±0.001	-63.3±0.635
3.	ETNLE 3	202.7±1.13	0.435±0.006	-43.1±0.529
4.	ETNLE 4	203.4±1.18	0.425±0.012	-27.9±0.451
5.	ETNLE 5	197.3±0.21	0.340±0.051	-12.7±1.266
6.	ETNLE 6	248.3±0.72	0.440±0.003	-47.8±0.252
7.	ETNLE 7	583.3±7.39	0.688±0.006	-82.9±0.513
8.	ETNLRP 1	353.4±0.25	0.647±0.014	-70.3±0.346

9.	ETNLRP 2	233.3±0.38	0.534±0.019	-64.5±0.700
10.	ETNLRP 3	229.2±0.40	0.518±0.006	-67.2±0.300
11.	ETNLRP 4	216.0±0.74	0.486±0.005	-69.9±0.624
12.	ETNLRP 5	260.7±0.34	0.672±0.001	-72.4±0.603
13.	ETNLRP 6	583.3±0.40	0.833±0.006	-75.1±1.513
14.	ETNLRP 7	448.6±0.51	0.586±0.002	-72.1±0.404



A- Formulation By Ethanol Injection Method



B- Formulations by reverse phase technique

Fig.10A- B: Particle size, PDI, Zeta potential, Drug loading, Entrapment efficiency measurement of ETNLE 5

2. Entrapment Efficiency and Drug Loading

Entrapment efficiency (EE) is defined as the ratio of drug in nanoparticles to the total amount of drug added to the formulation. The EE percent is the amount of drug encapsulated in the liposomal structure. In the development of liposomes as drug carriers, excellent encapsulation and retention of the encapsulated API are critical. A high drug-to-lipid ratio is likely to save formulation costs while simultaneously lowering the lipid-induced toxicity. The percentage of encapsulation was obtained based on the linearity graph of drug obtained by the HPLC method (mentioned above analytical method development and validation) standard curve of the drug's formulation.The percentage encapsulation efficiency and drug loading given in table 8. The % entrapment efficiency and % drug loading were range between 30.58± 0.67 to 81.78± 0.78% and 46.08±1.80 to 89.62±2.53%. The formulation ETNLE 5 showed better entrapment efficiency was found to be 81.78± 0.78%.

Table 8: Percentage Encapsulation Efficiency

Sr. No.	Formulation N=3	% Entrapment Efficiency	% Drug Loading
1.	ETNLE 1	47.43± 5.39	58.23±3.09
2.	ETNLE 2	51.70± 3.78	67.52±2.19
3.	ETNLE 3	57.18±3.71	75.83±1.12
4.	ETNLE 4	75.82± 2.87	78.61±0.51
5.	ETNLE 5	81.78± 0.78	89.62±2.53
6.	ETNLE 6	72.22± 4.02	74.20±2.53
7.	ETNLE 7	35.38± 1.97	71.43±2.42
8.	ETNLRP 1	30.58± 0.67	46.57±1.46
9.	ETNLRP 2	34.62± 2.01	46.08±1.80
10.	ETNLRP 3	50.49± 1.61	58.85±4.36
11.	ETNLRP 4	58.71± 1.06	70.33±0.23
12.	ETNLRP 5	61.48± 2.12	77.50±0.91
13.	ETNLRP 6	53.49± 1.91	59.82±3.06
14.	ETNLRP 7	34.69± 3.40	58.03±2.26

Evaluation of API and optimized formulation

1. In-vitro Drug Release

The drug release study of Etoposide and Nanoliposomal formulation (ETNLE 5) were performed by dialysis bag technique the results were shown in figure 11, 12 carried out at pH 1.2 and pH 6.8 respectively. In-vitro drug release studies were carried out for 60 minutes. Around 41% of drug was released in the first five minutes with a cumulative drug

released 63% for free ET. Around 15% of drug was released in the first five minutes with a cumulative drug released 58% for ETNLE 5 formulation at pH 1.2. The same formulation showed around 34% of drug was released in the first five minute with a cumulative drug released 71% for free ET. Around 21% of drug was released in the first five minute with a cumulative drug released 78% for ETNLE 5 formulation at pH 6.8.

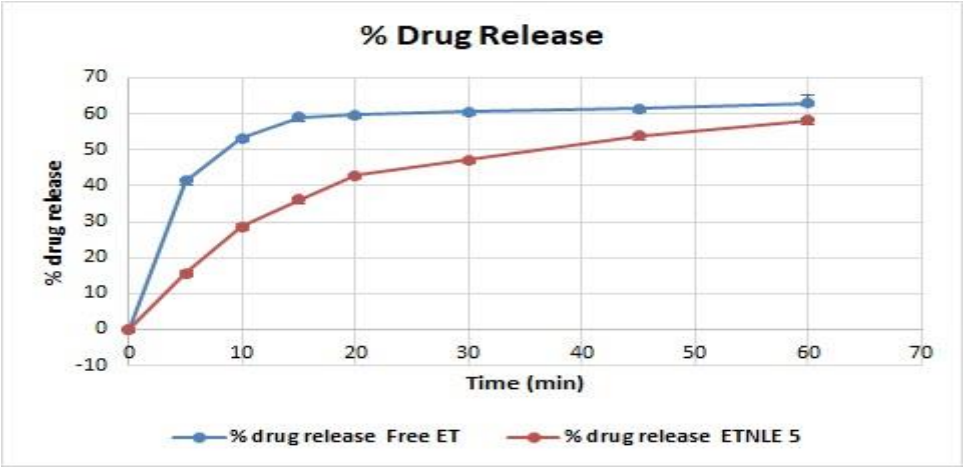


Fig.11: Comparative drug release of Free ET and ETNLE 5 at pH 1.2

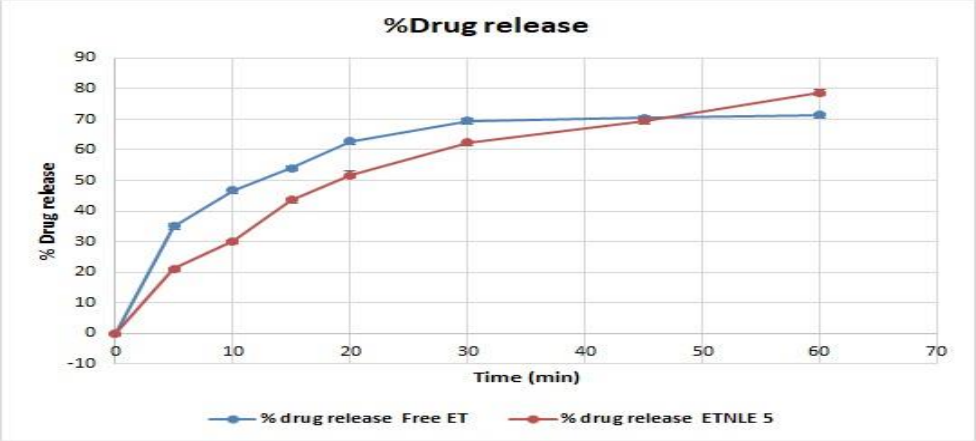


Fig.12: Comparative drug release of Free ET and ETNLE 5 at pH 6.8

1. Stability study

Stability study of optimized batch was performed for 1 year and parameters like particle size, entrapment efficiency and zeta potential were performed as function of time upon storage at 4 degree centigrade. Stability results were tabulated in table 9.

Table 9: Stability study of optimized batch ETNLE 5

Stability after month	Parameters		
	Particle Size (nm)	% Entrapment Efficiency	Zeta potential (mv)
Initial	197.1	81.78±0.99	-11.3
After 1 month	198.5	81.17±0.99	-11.9
After 3 month	201.1	80.62±0.99	-12.1
After 6 month	201.7	80.12±0.99	-12.3
After 9 month	204.2	79.91±0.99	-12.8
After stability study	205.1	79.86±0.99	-13.1

2. Cell viability studies

Cytotoxicity study was done on A-549 cells by MTT assay to express the influence of the new form of etoposide (Nano-liposomes) on induction of cell death. Figure 12 reveal that 42.26 % of cell viability of A-549 cells was inhibited by 200 microM equivalent etoposide liposomes as compared to 64.88% of free drug. These findings show that liposomal etoposide significantly exhibits increase in apoptotic cells as compared to other control groups.

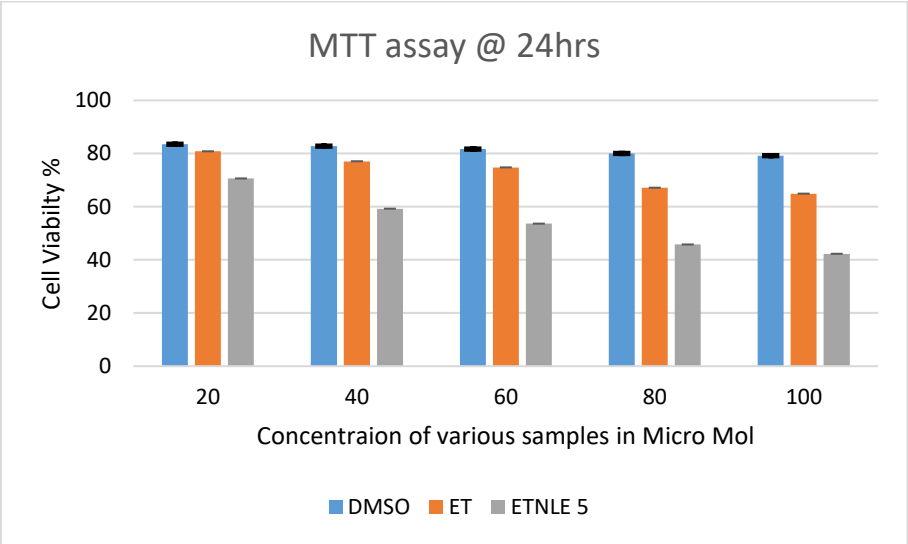


Fig.13: Graphs of MTT assay for determining cell viability for DMSO: Dimethyl sulfoxide; ET: Etoposide and ETNLE5:Etoposide Nano-liposome (NLs) Formulations by Ethanol Injection Method (5)

1. Surface morphology by TEM

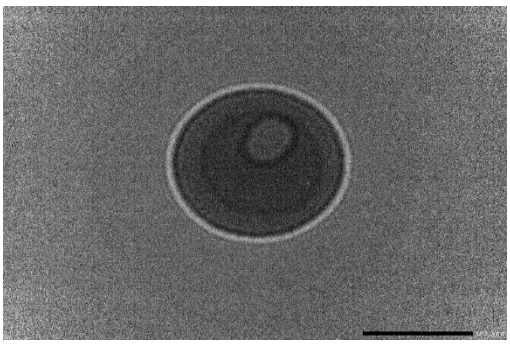


Fig.14: TEM images of ETNLE 5

The formed nanoparticle (ETNLE 5) was spherical with smooth surface, mono-dispersed pattern confirmed the slow release of the drug. The image was distinctly clear with a layered spherical structure with adequate size for desired bioavailability, stability and low toxicity.

2. Pharmacokinetic Study

Free etoposide and etoposide nano-liposomal formulation were administered orally with dose of 9 mg/kg (equivalent to free drug). The comparative pharmacokinetic parameters between free API and Liposome containing etoposide were calculated and are displayed in table 10.The concentration of drug was found to be higher in blood when the drug administered in the form of nano-liposome. Half-life (t1/2) of formulation 106.94 min was found to higher than API 65.92 min. Optimized formulation showed significant decrease in the Cmax. There was decrease in the clearance was observed. There was significant increase in the MRT was observed. The concentration of Etoposide- NL in the blood after oral administration (0.44 µg/ml) was significantly higher than API (0.43 µg/ml) at Tmax 1h. The increase in the concentration of Etoposide-NL in the blood was observed when compared with the API. The stable formulation exhibited delayed Tmax and also T1/2 rewarding the purpose of formulating liposomes. In our study as in many other cases [20, 34-39], due to the encapsulation of the drug, liposomes lower the free drug concentration in the blood and reduce the quantity of drug available for metabolism or renal clearance from blood. This increase the drug availability in the circulation and thus for target delivery.

Table 10: Pharmacokinetic study of formulation

Sr. No.	Parameter	Etoposide	Nano-liposomal formulation
1.	t1/2	65.91717092 min	106.9384315 min
2.	Tmax	60 min	60 min
3.	Cmax	0.443558172 µg/ml	0.439311817 µg/ml

t1/2: elimination half-life from 2-compartment model fitting; Tmax: time to reach maximum concentration; Cmax: maximum plasma concentration

3. Bio-distribution study

The tissue distribution of etoposide from nano-liposome formulation was determined after administration of the optimized formulations at a dose of 9 mg/kg given in figure 14. The average concentration of etoposide in drug loaded nanoliposome was found to be 4.52± 0.14 mcg/gm in liver, 20.39± 0.04 mcg/gm in lungs, 4.49± 0.14 mcg/gm in kidney and 7.53± 0.01 mcg/gm in spleen. The concentration of drug was too low in brain and heart to determine. The etoposide loaded nano-liposome (ETNLE 5) exhibited better drug targeting to lungs followed by spleen, liver and kidney. As expected, in liposomal formulation the nano size vesicles remained in circulation for prolonged periods of time and was in higher concentration in organs which are associated with macrophage phagocytic system (lungs, spleen and liver). The lower concentration in kidney suggests that the renal elimination is depleted. Overall, the liposomal structure of etoposide increases the bioavailability and organ targeted effect.

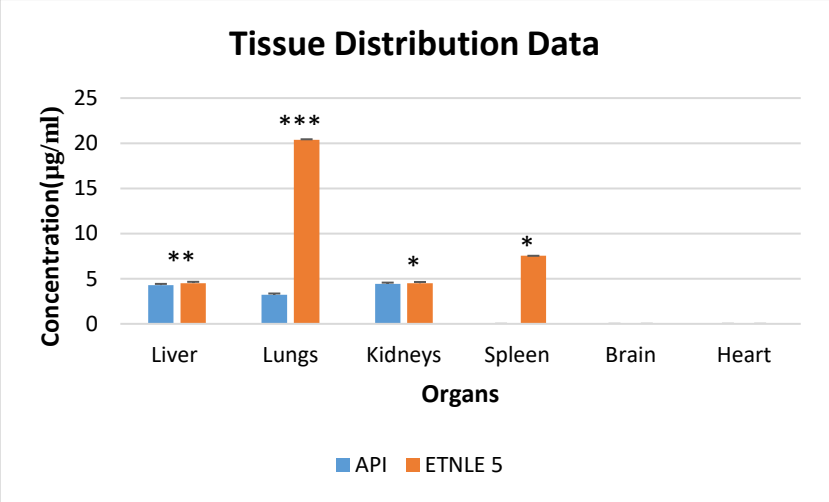


Fig.15: Bio-distribution Study

ND- Not determined

*- p < 0.001 - highly significant

**- p < 0.01 - Very significant

***- p < 0.05 - statistically significant

CONCLUSION

Lung cancer is a condition marked by uncontrolled cell proliferation in the lung tissue. If the condition is not treated, cell growth can expand out of the lung in a process known as metastasis and reach adjacent tissues or other organs. On the other hand, because lung cancer is latent during the first five years and the disease progresses when a substantial proportion of the cells are implicated in cancer, it is vital to develop treatment techniques to improve the performance of chemotherapy drugs, like etoposide, to have better treatment with minimal side effects.

Lipid-based nanoparticulate drug delivery system for anticancer drug was successfully developed and characterized. The nanoliposome developed by ethanol injection method and reverse phase evaporation techniques were evaluated for particle size, zeta potential, entrapment efficiency and in-vitro drug release. Formulated etoposide nanoliposome by ethanol injection method (ETNLE 5) showed good drug loading and entrapment efficiency. Drug release studies of nanoliposome showed promising results as compared to pure etoposide.

Overall, the study was extensive and completely evidently, to formulate and evaluate an effective and viable nano delivery system for etoposide. The nano size, anticipated zeta potential provided the non-aggregated formulation. The high efficiency of the liposomes aided the drug to be in the bi-layered liposomal structure even during circulating in the blood. This in-turn declined the renal elimination and enhances the availability of etoposide for the pharmacological action. The bio-distribution depicted that the concentration drug was higher in lungs which is again favorable for the better effect and lowering the dose of etoposide during lung cancer treatment. Thus, to conclude liposomal formulation by ethanol injection method with combination of DPMG-Na and CHO is an rapid and worthwhile drug delivery system for lipid loving drug like etoposide.

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