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Preparation, characterization, *in vitro* evaluation, cytotoxic and antitumor activity of rubitecan inlcusive liposomes with 2-hydoxy propyl-β-cyclodextrins as potential antitumor drug delivery system

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Abstract

It is an oral topoisomerase inhibitor, having oral absorption of about 25-30% leading to low bioavailability of the drug due to low permeability and poor water solubility. The aim of the present study is to improve the solubility and dissolution rate and in turn the sufficient activity against pancreatic cancer of the drug, by formulating its inclusion complex with beta (β)-cyclodextrin, using different methods. The phase solubility analysis indicates the formation of 1:1 molar inclusion complex of the drug with beta cyclodextrin. Phase solubility analysis of Rubetican-HP*β*CD mixture provided $k_{1:1}$ value of 1188 M^{-1}. The prepared complexes were characterized using differential scanning calorimetry, scanning electron microscopy, and x-ray diffractometry. The results suggest that using "drug-in-CD-inclusion complex in liposome" approach is a feasible strategy to formulate. These prepared liposomes by double-loading technique seem to be a suitable targeted drug delivery system because they have a fast onset action with prolonged drug release process and the significantly enhanced drug-loading capacity.

Keywords: 2-Hydoxy Propyl- β –Cyclodextrins, Inclusion complexes, Liposomes

Introduction

Nanocarriers are able to change the physicochemical properties of the incorporated molecules, affect the pharmacokinetic profiles of embedded drugs, as well as allow the incorporated molecules to overcome biological barriers. For these reasons nanovehicles are used to enhance the effectiveness of the drug, decrease severe side effects, and protect the drug from chemical degradation that may compromise their efficiency, or lead to the formation of secondary products, which are sometimes toxic. Liposomes are microscopic spherical lipid vesicles in which an aqueous volume is entirely enclosed by a membrane(1). They can encapsulate hydrophilic molecules in the aqueous core, lipophilic ones in the membrane and amphiphilic substances at the aqueous–lipid interface. Liposomes are usually made of natural, biodegradable, non-toxic and non-immunogenic lipid molecules. These aggregates are useful in biological, biomedical, and biotechnological applications as drug delivery systems due to their extraordinary capacity to encapsulate hydrophilic drugs in the aqueous core and to trap lipophilic compounds within the membrane. In doing so, this carrier system protects the entrapped molecules from degradation and thinning down in the systemic circulation. Because of their properties, liposomes when formed, their physiochemical properties like size, lamellarity, membrane rigidity, etc. are able to influence and enhance the performance of products by increasing ingredient solubility, improving ingredient bioavailability, enhance intracellular

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uptake, alter pharmacokinetics and biodistribution and *in vitro* and *in vivo* stability(2). Liposomes are also known to prevent local irritation, increase drug potency and reduce toxicity. However, the amount of drug that can be encapsulated within the liposomes impedes its employment as a carrier system. Both the properties of the liposome as well as that of the entrapped drug can manipulate the encapsulation efficiency. Liposomes offer an excellent opportunity to selective drug targeting which is expected to optimize the pharmacokinetic parameters and the pharmacological effects, prevent local irritation, and reduce the drug toxicity(2). Drugs may be incompatible with vesicle formation, and the accommodation of water-insoluble drugs in the lipid bilayers of the liposomes can be detrimental to the bilayer formation and stability and require the use of suitable organic solvents. Increasing the lipid load in order to incorporate sufficient drug for adequate therapeutic efficacy may not be acceptable, particularly with chronic use. Simultaneous entrapment of the drug into the lipid bilayers as well as into the aqueous phase of the liposome by virtue of the water-soluble cyclodextrin (CD) inclusion complexes is a prospective approach for surmounting such shortcomings and combining the relative advantages of the two types of carriers into a single system by formulating drug-in cyclodextrin-in liposome (CLDDS) systems(11).

Nevertheless, the incorporation of highly hydrophobic molecules into liposomes can destabilize the lipid membrane, leading to a rapid release of the drug from the bilayer. In an attempt to overcome this problem, in 1994, McCormack and Gregoriad is engineered, for the first time, the possibility of forming a combined system of cyclodextrins (CDs) and liposomes called drug-in-cyclodextrin-in-liposomes (DCLs). These systems are able to combine the capability of CDs to form CD/drug inclusion complexes with the use of liposomes as shuttle systems. Recently, different DCLs have been designed to overcome drawbacks such as the low solubility and low stability of many type of active compounds comprising aromatics, essential oils, and hydrophobic drugs. The possibility of using such a combined strategy aimed to concurrently exploit the CD-solubilizing power toward the drug and the tailored release mechanisms offered by liposomes.

Rubitecan [Orathecin™] is a topoisomerase I inhibitor extracted from the bark and leaves of the Camptotheca acuminata tree, which is native to China. It is a pyrano-indolizino-quinoline that is camptothecin in which the hydrogen at position 9 has been replaced by a nitro group. It is a C-nitro compound, a semisynthetic derivative, a tertiary alcohol and a delta-lactone. It is a prodrug for 9-aminocamptothecin. Investigated for use/treatment in pancreatic cancer, leukemia (unspecified), melanoma, ovarian cancer. Rubitecan is an effective drug against pancreatic cancer(4).

We decided to formulate inclusion complexes of 2-Hydoxy Propyl-β-Cyclodextrins containing Rubetican in liposome as the anticancer agent. However, the inherent bioavailability of the molecule is very low. This molecule has an aberrant solubility pattern and attempts to improve its bioavailability are plenty in the literature. A nanoparticulate delivery system for Rubetican has been designed; 2-Hydoxy Propyl-β-Cyclodextrins has been used as a complexing agent. Liposomes of Rubetican have been formulated as a stable carrier for delivering Rubetican to the hepatocytes is desired, warranting its delivery as CLDDS. All liposomal formulations were characterized for encapsulation efficiency, particle size, drug loading and morphology. *In vitro* release profiles were determined and an *in vitro* cytotoxicity and antitumor activity was performed to ascertain the effectiveness of the formulation developed(12).

Material

HP-β-CD (molecular weight, 1.460 Da) and dimethyl sulfoxide-d6 (DMSO- d6) were obtained from Sigma-Aldrich. The average degree of substitution in the purchased HPβCD was 0.8 hydroxypropyl groups per glucose unit.

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All other chemicals used were of standard reagent grade.

Preparation of Rubitecan Inclusion Complexes: Preparation of Rubitecan Inclusion is prepared by neutralization-agitation method: 1.500 g 2-Hydoxy Propyl-β-Cyclodextrins was weighed and placed into 50 mL beaker. 30 mL distilled water was added into the beaker to make a solution. Accurately weighed (0.3849 g) RUBITECAN (RUBITECAN to 2-Hydoxy Propyl-β-Cyclodextrins' molar ratio 1:1) was then added into the 2-Hydoxy Propyl-β-Cyclodextrins solution. 1 moL/L NaOH solution was then added dropwise to adjust the pH to alkaline. RUBITECAN, all in the open ring form, was dissolved in 2-Hydoxy Propyl-β-Cyclodextrins solution, followed by magnetic stirring for 2 h at room temperature. 1 moL/Litre hydrochloric acid solution was then dropped into the above solution to adjust the pH to neutral and kept overnight. The solution was then filtered and the filtrate was concentrated under reduced pressure at 40^0C vacuum drying. The RUBITECAN 2-Hydoxy Propyl-β-Cyclodextrins inclusion complex was then obtained and verified(4).

Liposome Preparation: Small uni-lamellar liposomes containing Rubitecan and Rubitecan: 2-Hydoxy Propyl-β-Cyclodextrins inclusion complexes were obtained by the method of formation-and- hydration-sonication of lipid film, as described by Lira et al. Briefly, to obtain Rubitecan liposomes, Rubitecan pure substance (10 mg) mixed with combined blend of lipids containing soya phosphatidyl choline, cholesterol and stearyl-amine) and was solubilized in the organic phase with the in a biphasic mixture solvent system composed of chloroform: methyl alcohol (3:1 v/v) under magnetic stirring. Next, a thin film was formed and hydrated with 10 mL of Tris buffer solution (pH 7.4) containing trehalose (10% m/v). The resulting multi-lamellar liposomes were then subjected to ultrasonication to obtain micro-uni-lamellar liposomes. Finally, the liposomes were freeze-dried and the lyophilized liposomes maintained at $25^oC \pm 1^oC$ in a desiccator equipped with vacuum control. Alternatively, in order to obtain Rubitecan inclusion complex form of liposomes, the Rubitecan: 2-Hydoxy Propyl-β-Cyclodextrins inclusion complexes were added to the Tris-buffer solution (aqueous phase) forming liposomes at a drug concentration of 1000 micrograms/mL(5).

Characterization of Inclusion Complexes in Liposomes:

Phase Solubility Test: It is commonly utilized to measure the binding constant calculated from the equations of Higuchi and Connors. Briefly, an excess of RUBITECAN (2 mg) was added in 2 mL of distilled water containing various amounts of HPβCD (0-200 mM). The dispersions were mixed by orbital stirring (Model TS2000A VDRL, Bio mixer, Brazil) at 150 rpm for 72 h at 25ºC±2ºC. Then, the samples were filtered through the hydrophilic cellulose acetate membrane (0.22 µm, Kasvi, Brazil). The RUBITECAN concentrations were determined spectrophotometrically at □ = 286 nm (model SP-2000 UV, Spectrum, USA). For Rubitecan, the apparent stability constant (Ks) was calculated from the linear relationship between the molar concentration of Rubitecan in the solution medium as a function of the HP-β-CD molecular concentration according to the following equation:

Slope- is the corresponding slope of the phase solubility diagram

$$Ks = \frac{Slope}{S_0(1\text{-}slope)}$$

S_0 (the intercept) denotes the solubility of Rubitecan in the absence of 2-Hydoxy Propyl-β-Cyclodextrins(13).

Morphological Studies: The morphology was evaluated and photo scanned by using scanning electron microscopy (SEM) (MEV-FEG Auriga 40, Zeiss, Germany) with an accelerating voltage of 5 kV. The test samples were previously covered with a layer of

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palladium by sputter coating. SEM reveals the inclusion cyclodextrins complexes to analyse whether the inclusion linkage had been completely obtained by comparing and contrasting the pictorial variations among the 2-HP-β-CD and final inclusion complex(6).

Particle Size Measurements: The average diameter of the liposomal particles and their polydispersity index (PI) of liposomes, at pre-and-post lyophilization/rehydration stage, were measured by photon-correlation-spectroscopic method (PCS) using a laser particle size analyzer Delsa-TM Nano-S at 25°C with a 90-degree fixed angle (Beckman Coulter, UK). Liposome samples (n = 3) were diluted suitably in highly purified water for particle counting.

Zeta Potential Measurements: The surface charge of the vesicles (zeta potential) was measured by determining their electrophoretic mobility using a Zetatrac Legacy (Nanotrac®, USA). The measurements (n = 3) were carried out at 25° C with sample dispersions at 0.3% (w/v) in a NaCl (1 mM) aqueous electrolyte.

Encapsulation Efficiency: It was estimated by UV-Visible spectrophotometry. The Rubitecan was determined by taking an aliquot of liposomal suspension (50 microliters). The aliquot was solubilized in methyl alcohol and is further subjected to sonication for 5 min to increase the solubility of aliquot in methanol. The analyte was then estimated by spectrophotometry at the absorption maxima of 276 nm (ʎmax), using the Rubitecan standard calibration curve with in working dilutions ranging from 2 to 20 micrograms/mL in methanol. To quantify the Rubitecan content that was not inclusively complexed into 2-HP-β-Cyclodextrins liposomes, the microfiltration/ dialysis/ultracentrifugation, a widely used technique, was used. Initially, an aliquot of liposomal dispersion (400 microliters) was centrifuged at 10,000 rpm for 20 minutes at 4°C. Next, the filtrate, whose free analyte in liposomes was quantified by UV-Vis spectrophotometry (276 nm). Dilution factors were applied to measure the correlation Entrapment efficiency was calculated by the following equation: EE% = (total-DCTN free Rubitecan)/total Rubitecan× 100%(7).

***In Vitro* Release Kinetics:** The kinetic profiles of Rubitecan liposomes and Rubitican: 2-Hydoxy Propyl-β-Cyclodextrins inclusion complexes encapsulated into liposomes in pH 7.4 Tris buffer solution for 1.5 days.

***In Vitro* Cytotoxicity and Antitumor Activity:** The cytotoxicity and antitumor activity were studied by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with various cancer cell lines (HCT116, KB), and L929 cells (regular homosapeins cell line). The cells were inoculated into 96-well plates and incubated for 24 hours at 37□ ambiences under controlled relative-humidity atmosphere in Dulbecco's Modified Eagle Medium-(DMEM) or Roswell Park Memorial Institute Medium-(RPMI 1640) with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) streptomycin sulphate. After 24-hours of inoculation cell agglomerates received drug, drug loaded in liposomes, inclusive complexes of drug and incubated for 48 h. After incubation, 30 µL of MTT solution (1000 ppm) was added to each well, mixed and incubated for 4 hours at 37°C. The media was withdrawn and then 100 microliters of DMSO was added to each well to and mix gently to solubilize the Formazan crystals developed by the viable and live cells. Further, the absorbance was measured at 550 nm (optical density) and 660 nm (for background subtraction). %-Cell viability was calculated according to the following equation: Optical Density- (OD) value = (OD 560 nm−670 nm)(8).

$$\text{Cell Viability (\%)} = \frac{OD_{Sample} - OD_{Blank}}{OD_{Control} - OD_{Blank}}$$

Cellular-Uptake Study by Confocal-Laser-Scanning-Microscopy: The cellular uptake of optimized and prepared inclusion complex is established by using the method confocal laser scanning microscopy in HCT-119 and KB cell linings. The HCT-119 and KB cells were inoculated on Octa-well plate with RPMI 1640 medium and incubated for

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24 hours. After incubation, the cells are treated with Rubitecan-2-HP-CD-inclusion complex and blend thoroughly for uniform distribution and then are incubated for 4 hours. The medium was collected and rinsed with Phosphate Buffer Solution for 2 times. Then the cell linings were stabilized by fixation with 4% paraformaldehyde for 10 min at 37°C. The micro-cellular machinery and other components including the nucleus of the cells were smeared and colour with Acti-stain-TM-488-phalloidin and DAPI for 15 min, and the slides were slightly treated with glycerol as dehydrating agent kept a side for 5 minutes. Further the slides are mounted and examined by CLSM(9).

Stability Assessment of Liposomes: These testing procedures include the physicochemical evaluations such as physical appearance, particle size measurement and distribution, poly-dispersity index and pH-variations were studied. The objective of accelerated stability study is to verify the liposomal preparations on short-term degradation by subjecting to stressed conditions. In this context, liposomal suspensions were undergoing the high-speed centrifugation at 10000 rpm for 1 hr at 4^{o}C and to mechanical stress at 180 strokes/minute at 37^{o}C during 48 hrs (Polytest®20 Bio-block Scientific). Long-term bench-top stability evaluation of liposomes was also recommended to perform immediately after the preparation of liposomes and subsequently at predetermined time intervals at 7, 15, and 30 days(10).

Results and Discussion

Phase Solubility Test: The concentration of RUBITECAN solubilized form in deionized water is raised in linear fashion with the addition of 2-Hydoxy Propyl-β-Cyclodextrins. The S_0 value (solubility of RUBITECAN in the absence of 2 HP-β-CD) was 0.000274 mM and the slope value of the linear plot was 0.00028, the slope value and S_0 value are important to obtain the stability constant (Ks) value. The determination coefficient was 0.9982, suggesting a equimolar ratio of 1:1 stoichiometry of the complex formation, and indicating an A_L-type phase solubility diagram according to Higuchi and Connors. In our study, the stability constant for the complexation was 1188 M^{-1}, the higher Ks value, the higher the chance of occurring an inclusion-complex between the active drug molecule and cyclodextrin and, henceforth, acquires the maximum drug solubility and successful complexation in the cyclodextrin cavity. The solubility profile of RUBITECAN in aqueous solutions containing 2-Hydoxy Propyl-β-Cyclodextrins (Figure 1).

Morphological Studies: The SEM images depicted that free Rubitecan is present as

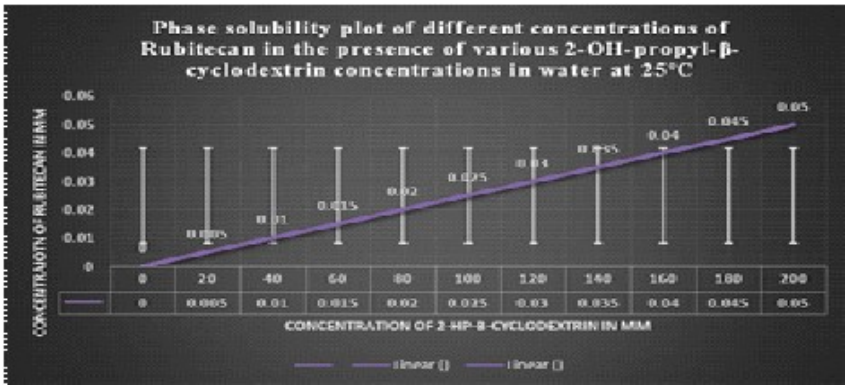

Fig 1. Phase Solubility Plot of Different Concentrations of Rubitecan in the Presence of Various 2-Hydroxy-Propyl β-Cyclodextrin Concentrations in Water at 25°C

Antitumor Drug Delivery System

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asymmetrical crystals with non-uniform shape and appearance. (Figure 2A) and 2-HP-β-CD are visible as spherical shaped particles with rough, uneven texture with high degree of crevices and pores on surface of the particles. (Fig. 2B). On the other hand, in the pictures of the Rubitican: 2-Hydoxy Propyl-β-Cyclodextrins inclusion complexes (Figure 2C) the intrinsic morphological characters of both molecules are modified and a uniform amorphous flat structure with highly variable in particle size distribution was observed. These variations in particle morphological features were attributed to the formation of linkage between the drug and cyclodextrins during inclusion complexation process, which suggests the formation of the inclusion complex.

Particle Size Measurements: The average particle sizes of plain liposomes and inclusive complexed cyclodextrin liposomes were measured to be 82.45 ± 6.6 nm and 87.33 ± 3.00 nm, respectively. There were no remarkable variations ($p<0.05$) in the equivalent mean diameters of particle sizes of plain and inclusive complex liposomes of Rubitecan. Also, the respective poly-dispersity index values of plain and inclusive complex liposomes of Rubitecan were found to be 0.35 and 0.39. The liposomes are ultimately had a uniformly distributed and populated particles.

***In Vitro* Release Kinetics:** A quick release effect (25%) to native liposomes and cyclodextrins liposomes occurred in the first four hours. This might be attributed to the quick release of Rubitecan adsorbed on the surface of liposomes. Henceforth, a slow and persistent release of the Rubitecan has observed in both formulations within a period of 6 hours (4–10 h). After 8 h, there was a drug release of almost 80% to both plain and cyclodextrin liposomes. The highest concentration of drug released to Rubitecan (95%) and to Rubitican:2-Hydoxy Propyl-β-Cyclodextrins inclusion complexes (98%) was obtained at 24 h. As shown in Figures (a) and (b), there was substantial agreement about the finding indicates that a simple diffusion mechanism is involved in the mass transport of liposomal systems Figs 3-10.

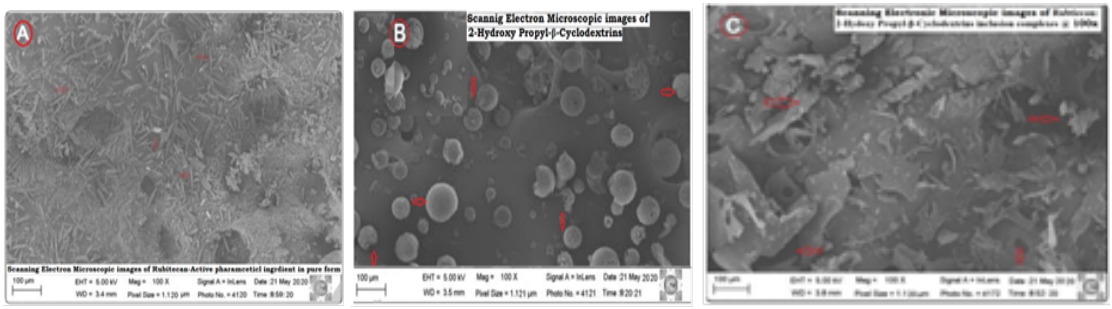

Fig 2. (A) Scanning Electron microscopy, (B) SEM Image, (C) SEM Image (SEM) of Rubuitecan-API in pure form

Table 1: Illustrates Particle Size, Zeta Potential and Encapsulation Efficiency

Liposomes	Particle Size in nm	Poly Dispersity Index	Zeta Potential in mV	Encapsulation Efficiency
Rubitecan Liposomes (Native)	73.65±5.24	0.31±2.78	36.45±2.74	94.72±2.64
Rubitecan: 2-HP-CD-Inclusive Complex Liposomes	88.58±2.84	0.39±6.85	38.76±3.65	92.18±3.42

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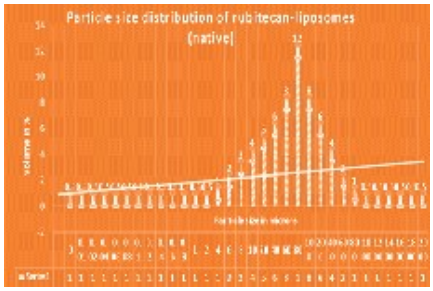

Fig 3. Particle Size Distribution of Rubitecan Liposomes

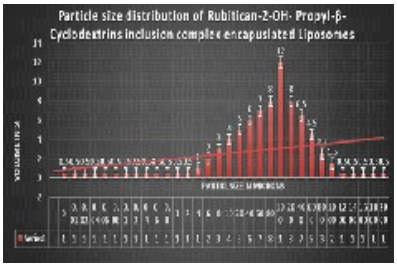

Fig 4. Particle Size Distribution of Rubitecan-2-Hydroxy Propyl-Beta-Cyclodextrins Inclusion Complexes Encapsulated Liposomes

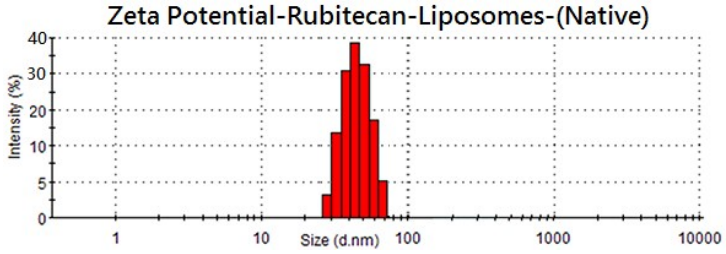

Fig 5. Zeta Potential Study of Rubitecan Liposomes

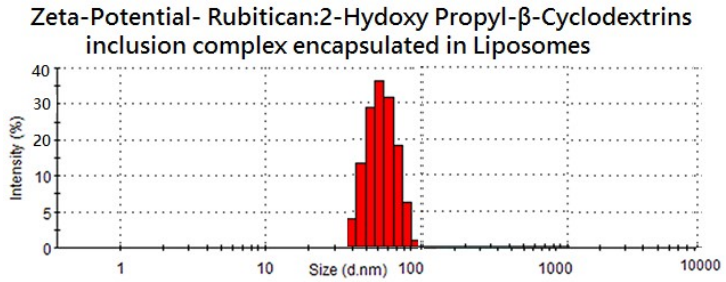

Fig 6. Zeta Potential Study of Rubitecan-2 Hydroxy Propyl-Beta-Cyclodextrins Inclusion Complexes Encapsulated Liposomes

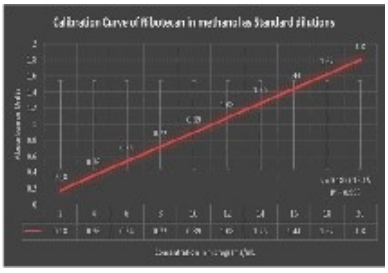

Fig 7. Calibration Curve of Rubitecan In methanol as standard dilutions

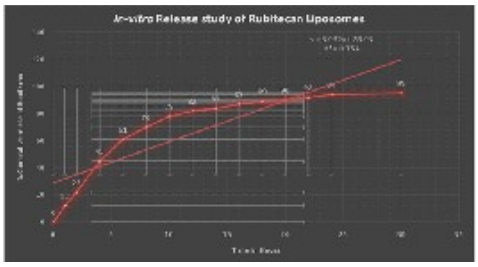

Fig 8. *In Vitro* Release Study of Rubitecan Liposomes

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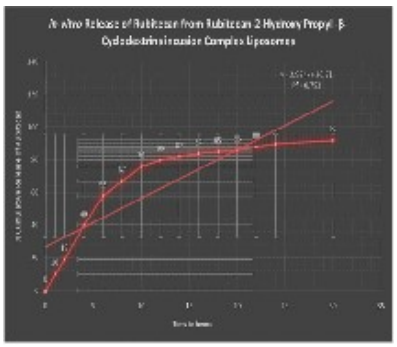

Fig 9. *In Vitro* Release Study of Rubitecan-2-Hydroxy propyl-Beta-Cyclodextrins Inclusion complexes

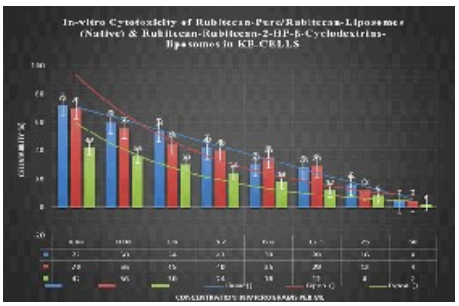

Fig 10. *In Vitro* Cytotoxicity Study of Rubitecan (Pure)/Rubitecan 2-Hydroxy propyl –Beta Cyclodextrins Inclusion Complexes in KB Cells

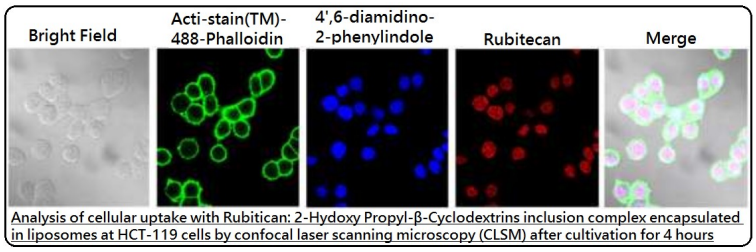

Fig 11. Analysis of cellular uptake with Rubitecan-Hydroxy propyl -Beta-cyclodextrins Inclusion Complexes at KB cells by Confocal laser Scanning Microscopy (CLSM) after Cultivation for 4 Hours

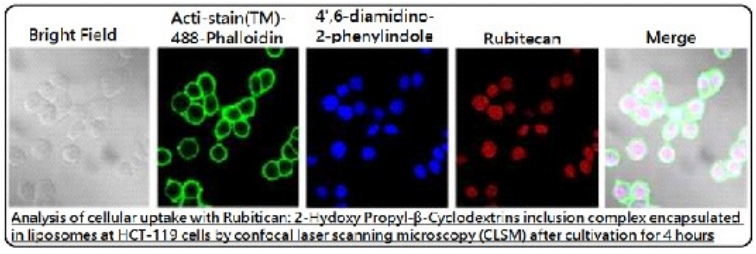

Fig 12. Analysis of Cellular Uptake with Rubitecan-2-Hydroxy Propyl-Beta-Cyclodextrins Inclusion Complexes at HCT-119 Cells by CLSM after Cultivation for 4 Hours

***In vitro* Cytotoxicity and Antitumor Activity:** The cytotoxicity and antitumor activity of free Rubitecan, Rubitecan-loaded-liposomes, and Rubitican: 2-Hydoxy Propyl-β-Cyclodextrins inclusion complex encapsulated in liposomes were evaluated by MTT assay [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] HCT119 and KB cell (cancer cell) (Figure). Viable cells at the time of test agent addition (T0) and following 72 hours of drug exposure were determined. In addition, anticancer activity shown (Fig. (B), (C)) that anticancer activity of Rubitecan-inclusion complex was improved than free Rubitecan and Rubitecan-loaded-liposomes in HCT 116 and KB cell Figure 11-13.

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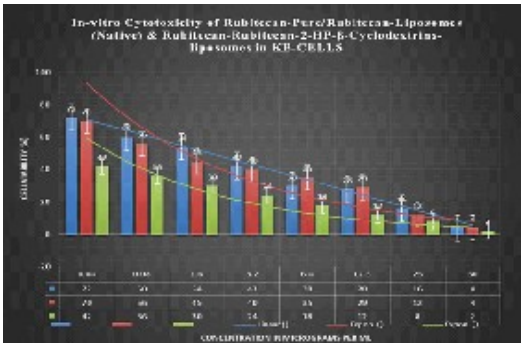

Fig 13. *In Vitro* Cytotoxicity Study of Rubitecan (Pure)/Rubitecan-Lipsomes/Rubitecan-2-Hydroxy Propyl-Beta-Cyclodextrins Inclusion Complexes in HCT-119 Cells

Conclusion

The outcomes exhibit that liposomal dosage forms containing Rubitecan and its incorporation complex were acquired with high entrapment efficiency. The details additionally stayed stable when submitted to both short term and long-term stability tests introducing and keeping up attractive physicochemical features for example, particle-size, polydispersity indices and zeta-potential, for intravenous use. Along these lines, the improvement of liposomal formulations containing Rubitecan and its incorporation complex Rubitecan: HP-β-CD speaks to a significant development for the utilization of this medication treatment reflecting a scientific approach and could be a likely choice in malignant growth treatment.

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