RESEARCH ARTICLE

Formulation and Evaluation of Rivaroxaban Loaded Liposomes

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ABSTRACT

The advanced delivery system is required formulation of rivaroxaban loaded liposomes in the context of highest solubility were observed in organic solvent DMSO and lower in distilled water, and 6.8 pH buffer shown greater solubility of drug. Absorbance of each solution was measured at 295nm using Shimadzu UV-1800 Spectrophotometer using pH 6.8 phosphate buffers as a reference standard. The calibration curve was prepared for solutions of 5µg/ml to 30µg/ml. It was observed that the absolute values of zeta potential were lower than those values reported in the literature that negative charge particles do not affect the stability of formed liposomes. Based on the rank order performed for all conventional RIV liposomes formulae depending on their characterization and evaluation tests, one optimized liposome formulation were selected. From the *in* vitro drug release data, drug loading efficiency and particle size analysis formulae F4 were selected as optimized formulation. The drug content in F-4 formulation was almost identical with the results so there was no change of percentage drug content after formation of RIV liposomes. In each situation, R^2 value was determined from the chart and Zero order release kinetics was found (R^2=0.992) to fit the release data best.

Keywords: Rivaroxaban; Liposomes; RIV liposomes, Chitosan coating

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INTRODUCTION

Liposomes are most often composed of phospholipids especially phosphatidylcholine, and cholesterol but may also include other lipids,[1] such as those found in egg and phosphatidylethanolamine, as long as they are compatible with lipid bilayer structure. A liposome design may employ surface ligands for attaching to desired cells or tissues. To deliver the molecules to a site of action, the lipid bilayer[2] can fuse with other bilayers such as the cell membrane, thus delivering the liposome contents; this is a complex and non-spontaneous event, however that does not apply to nutrients and drug delivery.[3] By preparing liposomes in a solution of DNA or drugs (which would normally be unable to diffuse through the membrane) they can be (indiscriminately) delivered past the lipid bilayer. Liposomes can also be designed to deliver drugs in other ways. Liposomes that contain low (or high) pH can be constructed such that dissolved aqueous drugs will be charged in solution (i.e., the pH is outside the drug's pI range).[4] As the pH naturally neutralizes within the liposome (protons can pass through some membranes), the drug will also be neutralized, allowing it to freely pass through a membrane. These liposomes work to deliver drug by diffusion rather than by direct cell fusion. However, the efficacy of this pH regulated passage depends on the physiochemical nature of the drug in question (e.g. pKa and having a basic or acid nature), which is very low for many drugs.[5]

We searched the approved drug database published on the website of the FDA and EMA, and found that 14 types of liposomal products have been authorized.[6] It should be noted that this list excludes generics, lipid complexes (e.g., Abelcet, Amphotec, and Onpattro), and nationally authorized liposomal products in Europe. Doxil (Doxorubicin HCl liposome injection) was the first liposomal product approved by the FDA in 1995. Among these marketed products, 43% of products were approved before the year 2000, and 57% of products were approved before the year 2010.[7]

MATERIALS AND METHODS

The pure drug rivaroxaban procured from Neuland laboratory, Hyderabad, cholesterol (97%), chitosan, tween 80, span80, macrogol 3500 were obtained from chemdyes Pvt. Ltd. Gujarat.

Preformulation Studies

Drug-Excipients Compatibility Studies

Drug-excipients compatibility studies were carried out using FTIR spectrometer (Model FTS-14).

Determination of Melting Point of Rivaroxaban

The melting point of rivaroxaban was determined using a digital systonic melting point apparatus (model No. S-973).

Solubility studies

Solubility of the drug rivaroxaban was checked in different solvents (distilled water, ethyl alcohol, DMSO, Different pH buffer from 1.2 to 7.4 range were checked by taking a fixed amount of drug and it was dissolved in fixed volume of solvents.[8] Amount of drug dissolved was evaluated by Shimadzu UV-visible spectrometer (model No. SL-1800).

Determination of λ_{max}

100 mg of the drug was accurately weighed and dissolved in sufficient quantity of Phosphate buffer (pH 6.8) and the

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final volume was made up to 100 ml to prepare the stock solution of solution of 1000 µg/ml. From the stock solution 1 ml was withdrawn and volume was made up to 100 ml with Phosphate buffer to obtain the solution of 10 µg/ml.[9] The resultant solution was scanned from 200 to 400 nm and the spectrum was recorded to obtain maximum wavelength $\lambda_{max.}$

Formulation of Trial Batches

Preparation of Rivaroxaban Loaded Liposomes

Preparation of Liposomes was done using lipid film hydration method (Bangham method). Weighed quantities of rivaroxaban, and mixture of lecithin and cholesterol were dissolved in 10 mL of chloroform then mixture of span 80 tween 80 and macerogol were added as per given quantities. The clear solution was dried along the inner walls of an 800 mL evaporating flask using rotary flash evaporator at 50-55°C under vacuum. After complete

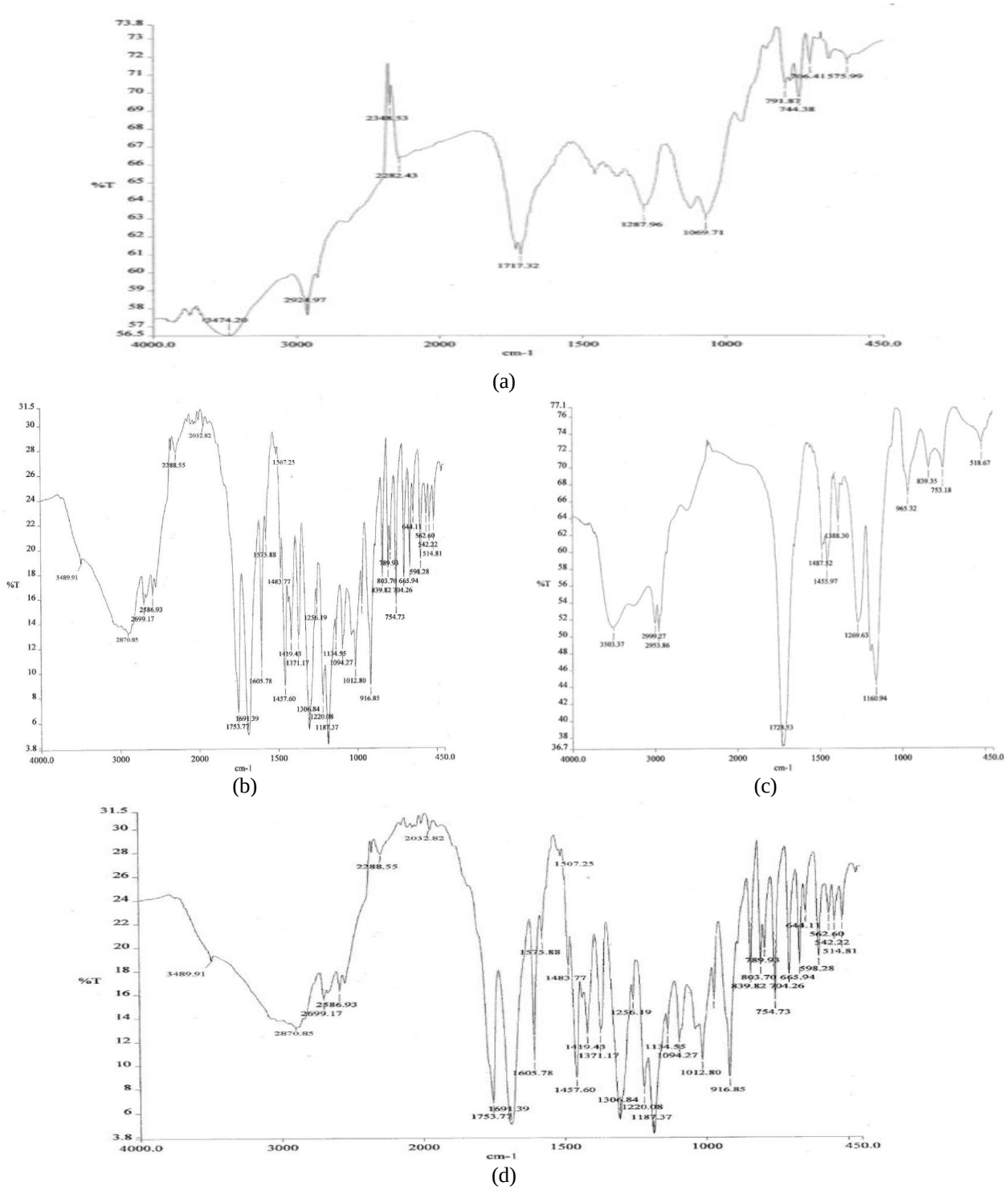


Figure 1: FTIR spectra of (a) Pure Rivaroxaban; (b) Drug, Lecithin; (c) Drug, Cholesterol; (d) Physical mixture of Drug, Lecithin and cholestero

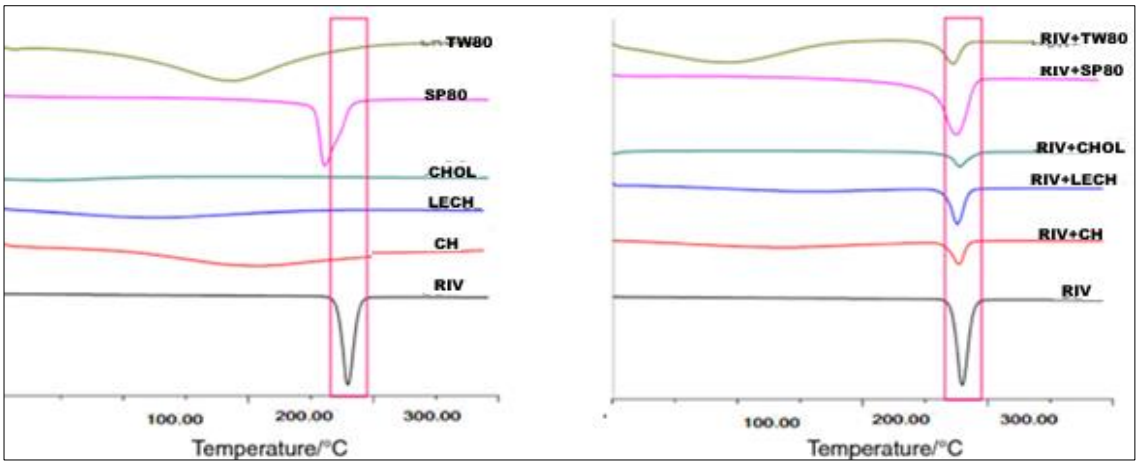


Figure 2: DSC thermograms of pure RIV and with excipients

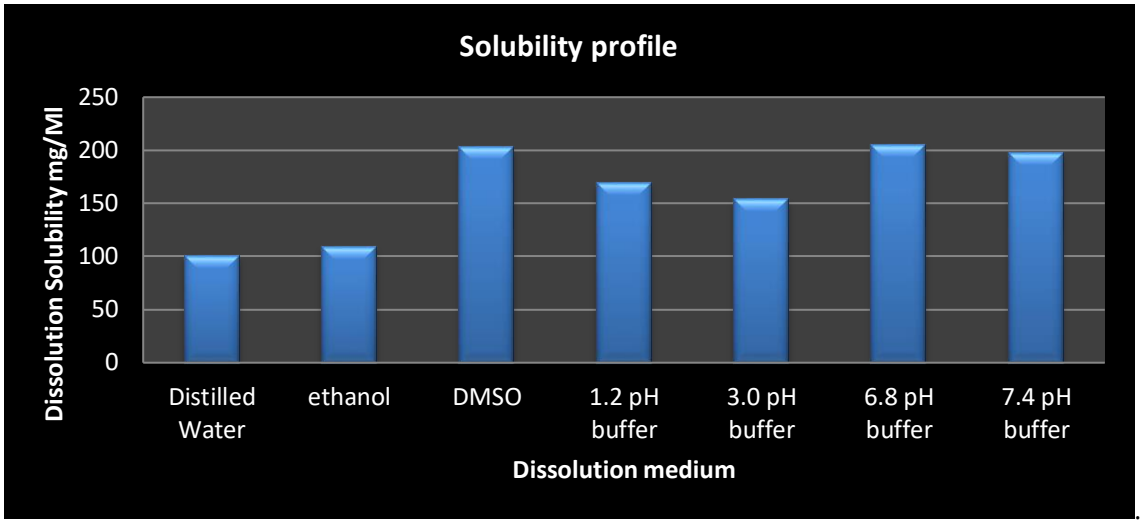


Figure 3: Solubility profile of rivaroxaban

removal of the solvent, the flask was kept overnight in a desiccator for further drying of the film. The dried lipid film was then hydrated with 10 mL phosphate buffer pH 7.4 warmed to 60-70°C (above the transition temperature of the lipid) and swirled for 15 min to obtain a heterogeneous system of vesicles in the medium. Finally, the vesicles were sonicated using a Vibracell sonics probe sonicator at 35% amplitude with 50 sec ON and 10 sec OFF cycles for 5 minutes. The size reduced vesicles were refrigerated at 4°C until further use. The volume of liposomal dispersion was 10 mL containing 10 mg of rivaroxaban.[10]

Coating Liposomes with Chitosan

Chitosan was solubilized in 0.5 %v/v acetic acid solution by stirring for 24 hrs with occasional warming. Liposomal dispersion was added gradually to an equal volume of Chitosan solution dropwise under magnetic stirring. Stirring was maintained for 15 min after which the coated liposomes were refrigerated overnight for stabilization of

Table 1: Trial Batch 1

Ingredients in %	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9
RIV	1	1	1	1	1	1	1	1	1
Chitosan	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Lecithin	20	20	20	20	20	20	20	20	20
Cholesterol	05	05	05	05	05	05	05	05	05
Span 80	1.00	1.00	1.00	0.75	0.75	0.75	0.50	0.50	0.50
Tween 80	0.70	0.50	0.20	0.70	0.50	0.20	0.70	0.50	0.20
Macrogol3500	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Ether	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Methanol	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
chloroform	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phos. Buffer pH 6.8	05	05	05	05	05	05	05	05	05

Table 2: Trial Batch 2

Ingredients in %	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9
RIV	1	1	1	1	1	1	1	1	1
Chitosan	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Lecithin	20	20	20	20	20	20	20	20	20
Cholesterol	05	05	05	05	05	05	05	05	05
Tween 80:Span 80	0.70 :1.0	0.50 :0.75	0.20 :0.50	0.70 :1.0	0.50 :0.75	0.20 :0.50	0.70 :1.0	0.50 :0.75	0.20 :0.50
Macrogol 3500	1.0	1.0	1.0	0.75	0.75	0.75	0.50	0.50	0.50
Ether	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Methanol	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
chloroform	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phos. Buffer pH 6.8	05	05	05	05	05	05	05	05	05

X_1= low (0.50), medium (0.75), High (1) level in % (Macrogol 3500)
X_2= low (0.50:0.20), medium (0.70:0.50), High (1:0.70) level (Tween 80: Span 80)

the Chitosan coat. Then the coated liposomes were centrifuged and washed with 0.5% v/v acetic acid several times to remove the excess chitosan. Finally, the coated liposomes were dispersed in distilled water and stored in a refrigerator (4 °C) until further use.[11]

Percentage Drug Entrapment Efficiency

Percentage EE of liposomes 1mL of liposomal dispersion was centrifuged at 15,000 rpm using a refrigerated ultracentrifuge (REMI) at 4°C for 10 min. The pellet consisting of the entrapped drug was lysed with acetonitrile for 10 minutes using a bath sonicator to release the drug. It was again cold centrifuged for 10 min at 15,000 rpm, and the supernatant was scanned for the entrapped drug (C) using HPLC Total drug (Cd) in the aliquot was evaluated by lysing vesicles present the 1 mL of dispersion with acetonitrile followed by ultracentrifugation and analyzing

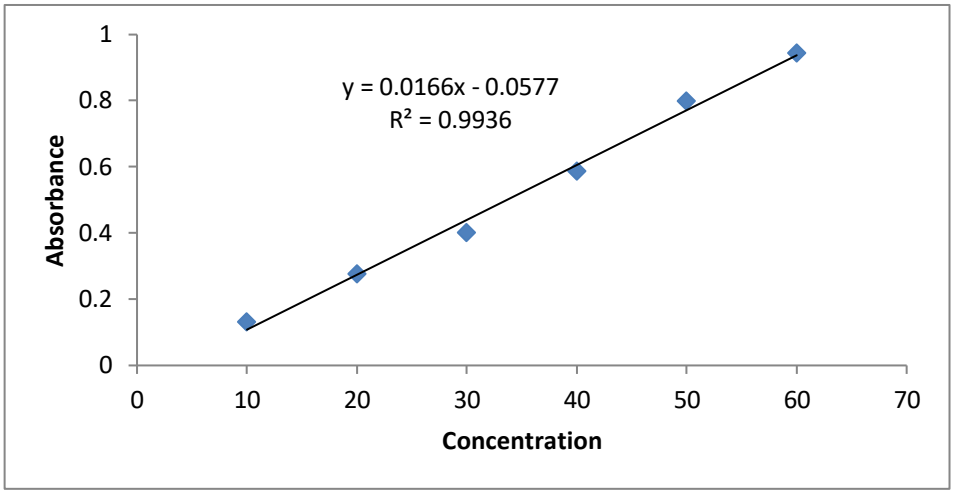


Figure 4: Drug calibration curve in phosphate buffer pH 6.8

Figure 5: UV spectrum of RIV in 6.8 phosphate buffer

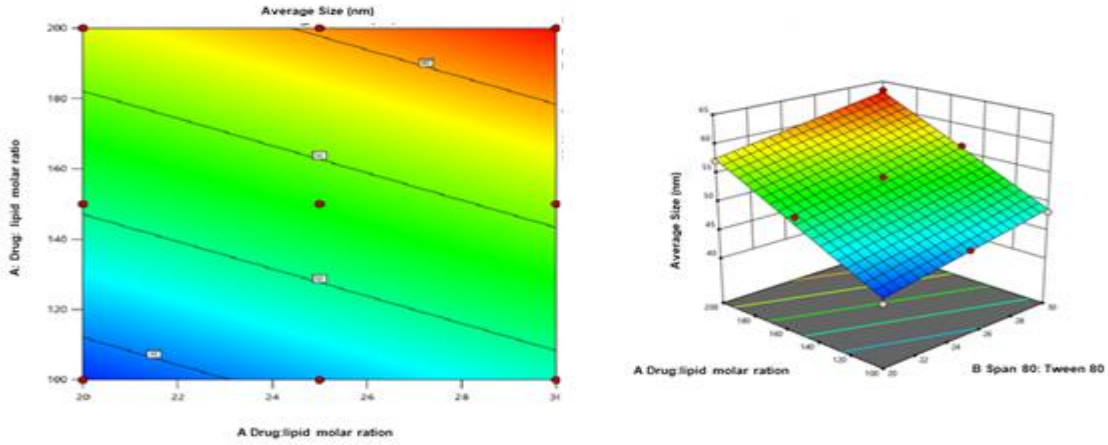


Figure 6: Response surface (A) and its Contour plot (B) shows effect of X_1 and X_2 on vesicle size

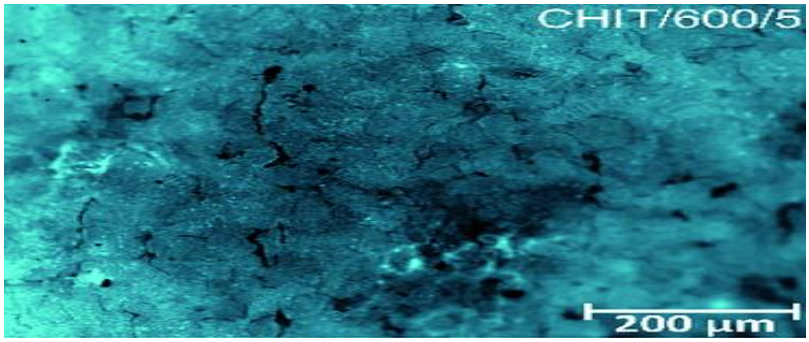


Figure 7: Fluorescence photomicrographs of chitosan

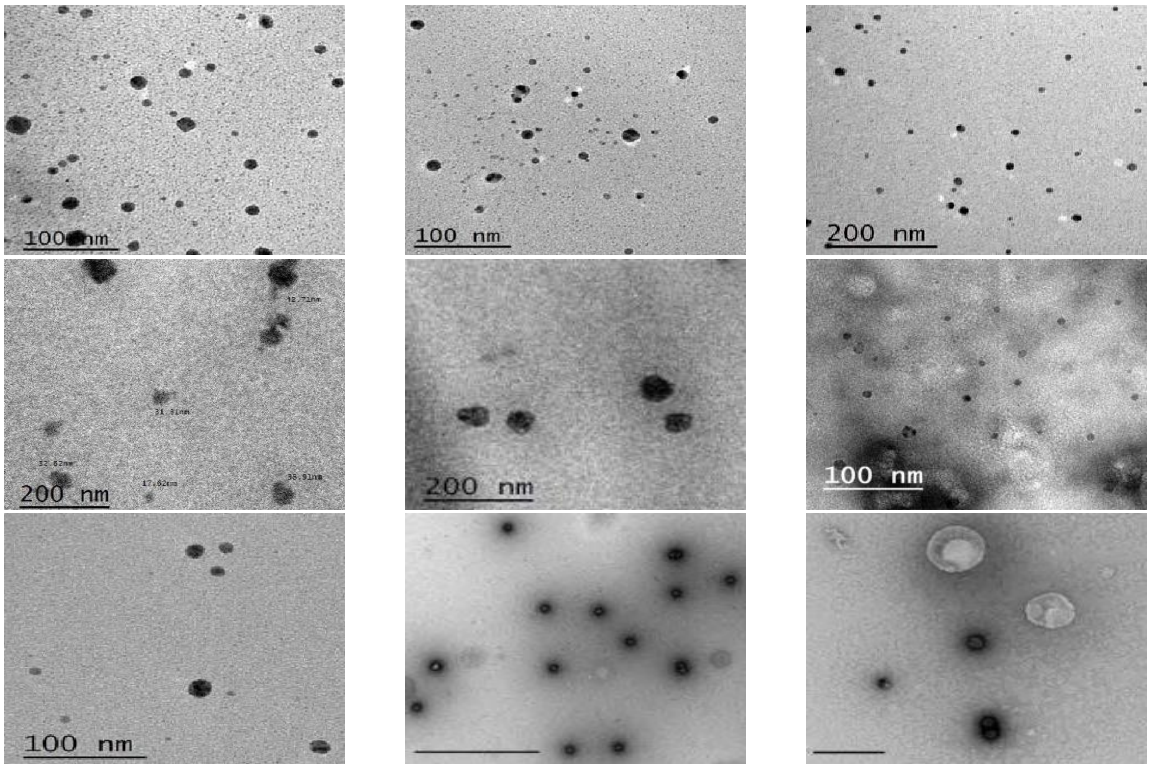


Figure 8: TEM photographs of RIV loaded Liposome formulations of F1-F9

the supernatant drug concentration. %EE was calculated using equation.[12]

$\%EE = [C/Cd] \times 100$ (2)

Statistical Analysis and Optimization of the Batches

To study the effect of variables on liposome performance and characteristics, different batches were prepared using 3^2

Table 3: % selection of Variable X_1 and Variable X_2 (Trial 1)

Batch Code	Variable X_1 amount of span 80 (%)	Variable X_2 amount of Tween 80 (%)
F-1	+1 (1)	+1(0.70)
F-2	+1 (1)	0 (0.50)
F-3	+1(1)	-1 (0.20)
F-4	0 (0.75)	+1(0.70)
F-5	0 (0.75)	0 (0.50)
F-6	0 (0.75)	-1(0.20)
F-7	-1 (0.50)	+1 (0.70)
F-8	-1 (0.50)	0 (0.50)
F-9	-1 (0.50)	-1 (0.20)

Note: + High level; 0 Medium level and -1 as Low level

factorial designs. Amount of Span 80 and Tween 80 were selected as two independent variables. Vesicle size and %EE in Amount of Cholestrol and Licithin were kept constant.[13]

Response surface plot Contour plot and (3D) response surface plots were constructed to establish the understanding of relationship of variables and its interaction.

Evaluation of Formulation Batches

Determination of Vesicle Size, Zeta Potential (ZP) and Percentage Entrapment Efficiency

Uncoated liposomes, Chitosan coated liposomes (CCLs), was evaluated for zeta potential and mean size using Malvern-Nanoparticle size analyzer using of dynamic light scattering (DLS) and electrophoretic mobility of particles. To evaluate the %EE, 1 mL samples of both the single and dual coated liposomes were centrifuged for 15 min at 15,000 rpm at 4 °C. The pellets were ultrasonicated with 10 mL acetonitrile for 15 min and centrifuged to separate the polymer and lipid debris from the free drug released in the supernatant. The drug concentration was analyzed from the supernatant using HPLC, and the percentage entrapment efficiency was determined.[14]

Visualization of Chitosan Coating by Fluorescence Imaging

Liposomes were prepared using the earlier reported method with sodium fluorescein added in the chitosan solution. Post-coating, the excess fluorescein was removed by repeated centrifugation and washing of the pellet with distilled water. The dispersion was spotted on a glass slide and sealed with a coverslip. The fluorescence photomicrographs were obtained using a Leica DMC 4500 fluorescence microscope using 100X objective.[15]

Surface Morphology by SEM Imaging

Uncoated liposomes were freeze-dried for 48 hours whereas the liposomes were dried for 48 hrs using hot air oven at 40°C. The residues were further dried for 24 hrs in a desiccator and subjected to SEM imaging.[16] The samples were scattered on adhesive pads and placed on specimen mounts, sputter coated with palladium/gold and then scanned with the electron microscope (Zeiss Gemini).

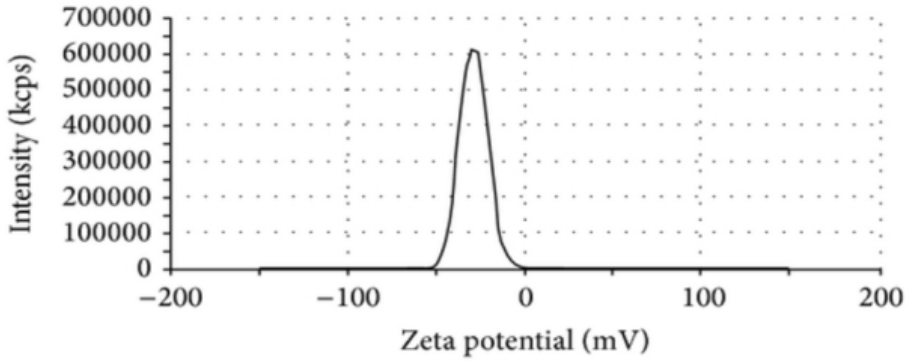
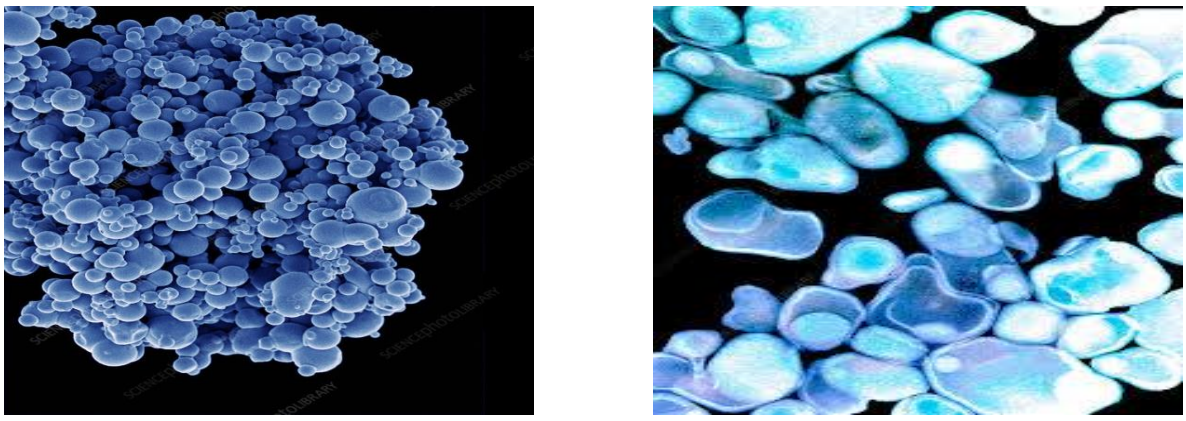


Figure 9: Zeta potential of F1-F9



(a) (b)

Figure 10: SEM photograph of F4 Liposome (a) Lower magnification (b) Higher magnification

Table 4: % selection of Variable X_1 and Variable X_2 (Trial 2)

Batch Code	Variable X_1 amount of Macrogol 3500 (%)	Variable X_2 amount of Tween 80 (%)
F-1	+1 (1)	+1(0.70)
F-2	+1 (1)	0 (0.50)
F-3	+1(1)	-1 (0.20)
F-4	0 (0.75)	+1(0.70)
F-5	0 (0.75)	0 (0.50)
F-6	0 (0.75)	-1(0.20)
F-7	-1 (0.50)	+1 (0.70)
F-8	-1 (0.50)	0 (0.50)
F-9	-1 (0.50)	-1 (0.20)

Transmission Electron Microscopy (TEM)

The morphology of Uncoated liposomes, Chitosan coated liposomes were contemplated by transmission electron microscopy (TEM) on a Philips EM268D instrument (Philips, Netherlands) at SAIF Panjab University, Chandigarh. The watery scattering of nanoparticles (one drop) was put more than a 400-network carbon-covered copper matrix followed by bad staining with phosphotungstic corrosive arrangement (3% w/v, acclimated to pH 4.7 with KOH) and put at the speeding up voltage of 95 kV for TEM.[17]

***In-vitro* Drug Release**

Selection of membrane chemistry: A RIV solution of 10 µg/mL in PBS buffer was filtered through different filters and assayed for recovery after filtration. 100, 300, and 500 kDa cross-flow filtration membranes were used for selection of the membrane pore size. RIV liposomes were pumped through the membranes and concentrated five-fold. The filtrate was then assayed for RIV content. The quantification of the release response time was realized by introducing dissolved RIV into the vessels shortly before the sampling point. The release response time of the NanoDis was qualified using RIV dissolved in DMSO.[18-23] The dissolved RIV was added to the vessels 5 seconds before each sampling time point.

Dissolution experiments were conducted with an Agilent 708-DS dissolution apparatus coupled with a NanoDis module and an Agilent 850-DS sampling station.

At predetermined time points, the samples were pulled from the vessel, filtered by the NanoDis module, and collected into vials by the 850-DS sampling station. The workflow was controlled by Agilent Dissolution Workstation software.

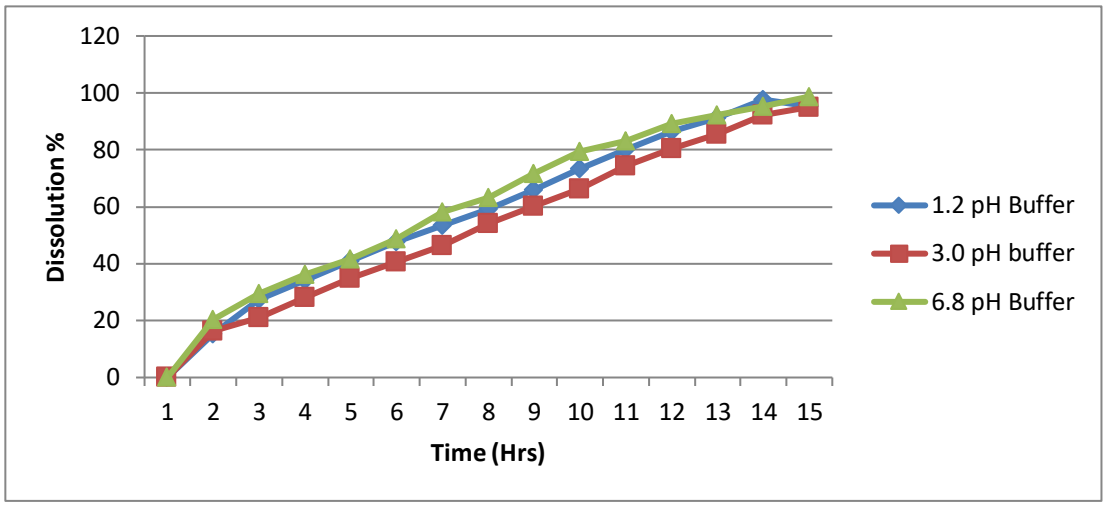


Figure 11: Drug release of RIV from liposome (F-4) in the different dissolution medium

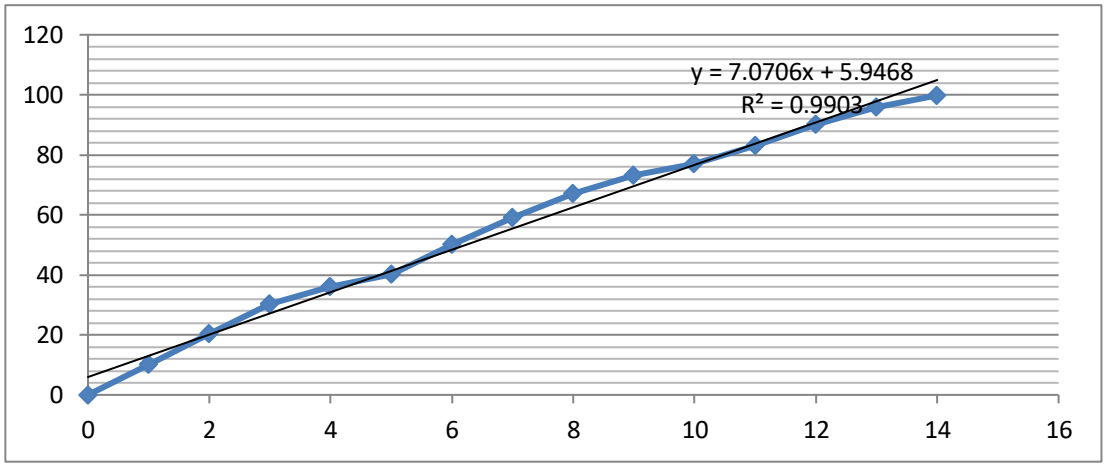


Figure 12: Zero order release kinetics of optimized F-4 formulation

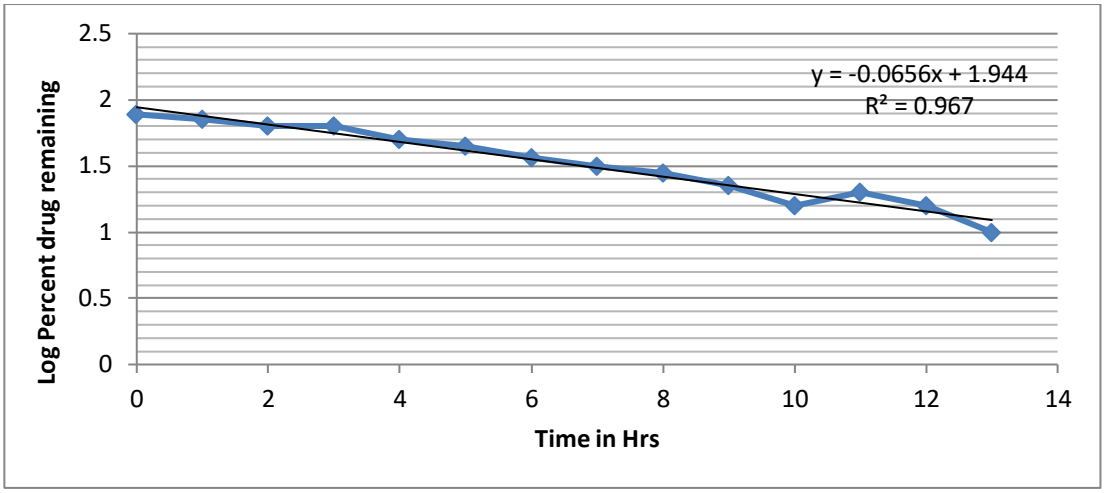


Figure 13: First order release kinetics of optimized F-4 formulation

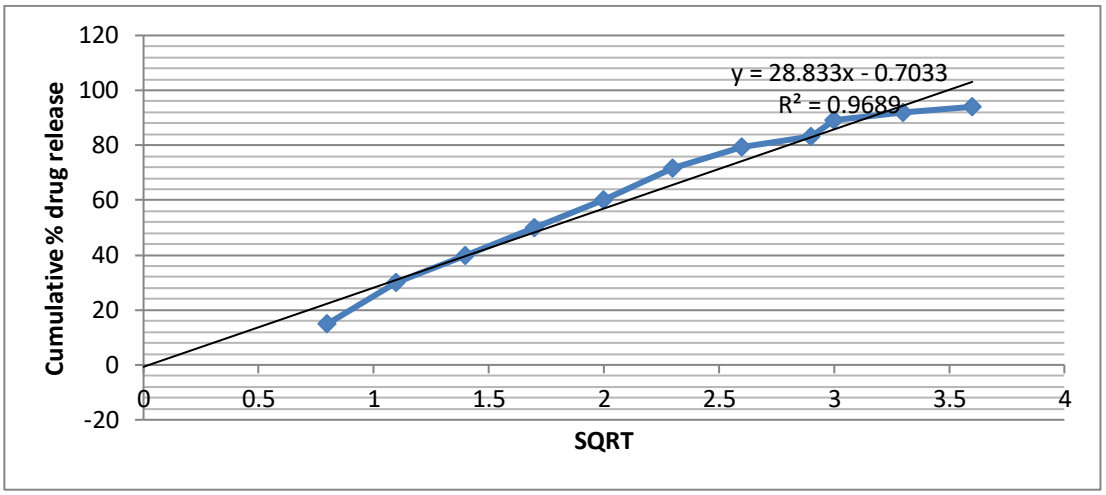


Figure 14: Higuchi's Model rrelease kinetics of optimized F-4 formulation

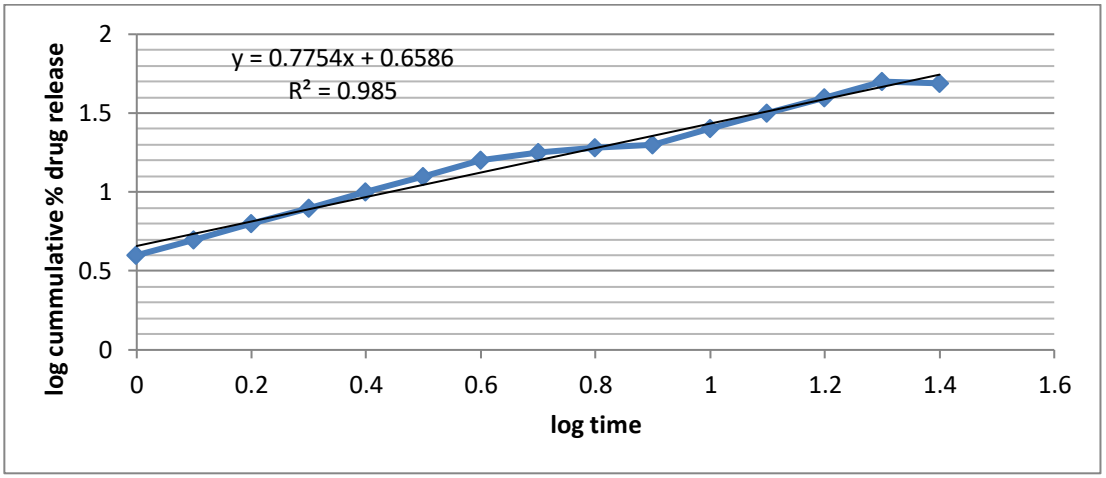


Figure 15: Korsmeyer-peppas release kinetics of optimized F-4 formulation

RESULTS AND DISCUSSION

Infra-Red absorption spectrum of the finely ground sample in KBr dispersion compressed into a disc should exhibit maxima only at the same wavelengths as that of a similar preparation of working standard.

Differential Scanning Calorimetry (DSC)

Thermograms of pure rivaroxaban, Lecithin, physical mixture of both Physical mixture of Drug, Lecithin and cholesterol were obtained using differential scanning calorimeter as shown in Figure 2. The thermogram of rivaroxaban exhibited a sharp endothermic peak at about

Table 5: Parameters of dissolution experiments

Dissolution Apparatus	USP II
Volume	900 mL
Rotation Speed	50 rpm
Sampling Time Points	5, 15, 30, 45, 60, 75, and 90 min
Buffer	pH 1.2, pH 3.0 and Phosphate buffer pH 6.8, pH 7.4

Table 6: Parameters for the Agilent NanoDis system

Operation	Setting
Peristaltic Flow Through Duration	240 s
Syringe Purge Volume	4 mL
Peristaltic Air Purge Duration	60 s
Pre Time Point Filter Conditioning	
Peristaltic Flow Through Duration	60 s
Syringe Purge Volume	2 mL

238.40^{O}C, corresponding to its melting point. It is known that transforming the physical state of a drug to the amorphous or partially amorphous state.

Solubility Studies

The highest solubility were observed in organic solvent DMSO and lower in distilled water, and 6.8 pH buffer shown greater solubility of rivaroxaban despite in figure 3.

Preparation of Calibration Curve of Rivaroxaban in Phosphate Buffer (pH 6.8)

Optimization of Formulation by Full Factorial 3^2 Design

Fitting the model to data Response data of all formulations were fitted to cubic, linear and quadratic model. According to design expert software, best-fitted model was linear for response Y_1 and quadratic for response Y_2. All the responses were fitted to model to establish full model (FM) polynomial equation.

$Y_1 = 964.78 + 113.\ X_1 - 169.83\ X_2 + 45.50\ X_1\ X_2 - 29.33\ X - 118.17\ X$

$Y_2 = 75.20 + 7.61\ X_1 + 4.64\ X_2 - 1.64\ X_1\ X_2 - 1.72\ X - 2.44\ X$

Response surface (3D) and Contour plot analysis. The obtained results can be observed visually in the response surface (3D) and contour plots (Fig 6). Response surface graph of Y1 shows that vesicle size of liposome was decreased with decreasing tween 80 concentration because phospholipids constitute the liposome membrane. With increasing total lipid Tween 80: span 80 concentration more drug could be incorporate into liposome. In addition, response surface graph of Y_2 shows that the increase in Tween 80: span 80 ratio significantly increased the drug entrapment efficiency. These results supported by the fact that, movement of fatty acids hydrophobic tails was reduced by incorporation of a bulky molecule of cholesterol in the lipid bilayer of liposome. It leads to permeability reduction of liposome membrane via resistance of phospholipids exchange with apoprotein. These ultimately improve the drug retention in liposome by prevention of drug leakage from lipid bilayer.

Transmission Electron Microscopy (TEM)

TEM photographs of RIV loaded Liposome formulations subsequent to post dilution with distilled water. TEM investigation uncovered that most plan showed round and homogeneous drops with a size more modest than 50 nm, which fulfils the rules of nanometric size range needed for liposomes recipe.

Table 7: Checkpoint batch with their predicted and observed value of responses

	Independent Variables		Vesicle size (Y_1)		Entrapment efficiency (Y_2)	
Batch	X_1	X_2	Observed	predicted	Observed	predicted
F-10A	0.089 (0.75)	+1(0.70)	647	653	78.90	74.67
Percentage prediction error (%)			0.92		+2.85	

Note: Independent Variables Vesicle size (Y_1) Entrapment efficiency (Y_2)

Table 8: Checkpoint batch with their predicted and observed value of responses

	Independent Variables		Vesicle size (Y_1)		Entrapment efficiency (Y_2)	
Batch	X_1	X_2	Observed	predicted	Observed	predicted
F-10	0 (0.50)	+1(0.75:1)	632	649	81.26	83.87
Percentage prediction error (%)			0.92		+2.61	

Table 9: Particle Size, %EE, Zeta Potential and Drug Loading Efficiency of Formulation

Formulation Code	Particle vesicle Size (nm)*	%EE	Zeta Potential (mV)*	Drug Loading Effciency*(%)
F1	677±0.20	80.20±0.02	-4.20±0.15	91.36±0.64
F2	819±0.30	67.31±0.07	-3.23±0.21	92.10±0.74
F3	820±0.18	82.20±0.04	-2.54±0.40	95.74±1.26
F4	771±0.12	83.05±0.01	-4.08±0.18	95.15±2.08
F5	640±0.15	82.96±0.04	-2.36±0.01	94.54±1.41
F6	762±0.21	81.60±0.04	-3.34±0.14	98.02±1.40
F7	956±0.44	76.60±0.01	-5.06±0.54	96.42±1.50
F8	705±0.39	79.70±0.04	-3.45±0.51	99.07±0.22
F9	605±0.43	73.03±0.01	-4.08±0.53	95.40±1.41

* Values are expressed as mean ± S.D., $n = 3$

Zeta Potential Determination

The zeta potential values of RIV loaded Liposomes formulation was in the range of -2.36±0.01to -5.06±0.54 Mv. It was observed that the absolute values of zeta potential were lower than those values reported in the literature that negative charge particles do not affect the stability of formed liposomes.

Drug Loading Efficiency

The drug loading efficiency for all RIV loaded Liposome formulation was found in the range of 91.36±0.64 (F1) to 99.07±0.22 (F9), showing uniform medication scattering in definition. Genuinely it was additionally legitimized that there was no critical contrast in drug content among the different definition. It was seen that equation F4 has the most noteworthy drug content.

Scanning Electron Microscopy (SEM)

The surface morphology of pure RIV powder and liposome formulation of RIV was determined using scanning electron microscope. The RIV powder appeared with an irregular crystalline shape as irregular and plate-shaped crystals having rough surfaces. The image of the RIV liposome formulation F-4 containing RIV however, illustrate that the particles had the same outer macroscopic morphology consisting of well separated spherical particles with relatively deep dents and similar diameters.

Drug Loading Efficiency

The amount of RIV present in the optimized F-4 formulation was found to be within the USP limit. The drug loading efficiency was found to be 96.23 ± 0.65 for formula F-4. The drug content in F-4 formulation was almost identical with the results so there was no change of percentage drug content after formation of RIV liposomes.

***In-vitro* Drug Release Studies**

Dissolution studies were conducted in 1.2, 3.0 and 6.8 pH buffer the drug release from the RIV liposomes. The drug release of RIV from the liposome formulation was dependent on the pH condition of medium the 6.8 pH medium shown as faster RIV releases from the RIV Liposomes (F-4) where 50% drug release was achieved within 6 Hrs. then followed by a constant slow release for the next 14 Hrs.

***In-vitro* Drug Release Kinetic of Optimized Formulation**

To understand the mechanism by which the medication was delivered from the RIV Liposomes formulation F1 and F9, various release kinetics model including zero order, first order, Higuchi and Korsmeyer-Peppas model were applied as shown in Figure.

CONCLUSION

Based on the rank order performed for all conventional RIV liposomes formulae depending on their characterization and evaluation tests, one optimized liposome formulation were selected. From the *in* vitro drug release data, drug loading efficiency and particle size analysis formulae F4 were selected as optimized formulation. The image of the RIV liposome formulation F-4 containing RIV however, illustrate that the particles had the same outer macroscopic morphology consisting of well separated spherical particles with relatively deep dents and similar diameters. The amount The drug content in F-4 formulation was almost identical with the results so there was no change of percentage drug content after formation of RIV liposomes. In each situation, R^2 value was determined from the chart and Zero order release kinetics was found (R^2=0.992) to fit the release data best. An *in vitro* study of the thrombolytic activity of liposomes was carried out using a similar procedure to that used for RIV. The results indicate that the formulation of RIV into liposomes doesn't result in a significant loss of activity *in vitro*. This might be due to the effect of polymers used in the formulation and the properties of liposomes like porous surfaces.

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