CHARACTERIZATION OF FORMULATIONS

6.1 Introduction

Adequate characterization of formulations is a prerequisite for quality control of the product. The QbD concept stresses the scientific understanding of characterization of formulation with acceptable stability profiles. Several parameters like zeta potential, in vitro drug release, % EE, vesicle size, morphology, drug loading were considered in present investigation for adequate characterization of liposomal carriers. Same methods of characterization were followed for all the three liposomal formulations viz. RLX loaded liposomes, LA loaded liposomes and dual drug RLX-LA loaded liposomes.

6.2 Materials and Equipment

Materials

Methanol and Chloroform (A. R. grade) was purchased from Merck, Mumbai, India. Sodium Hydroxide, Potassium Hydroxide, Calcium Hydroxide, Hydrochloric acid and Acetic acid were obtained from Loba Chemie, Mumbai, India. Bovine Serum Albumin, Lactic acid and Glycerol were procured from Merck Specialities Pvt. Ltd. Mumbai, India. Sodium Chloride, Urea and Glucose were purchased from Qualigens Fine Chemicals, Mumbai, India. Distilled water used in the study was filtered using 0.22 micron nylon filter, Nylon N66 membrane filters 47 mm, Rankem, India. Cellulose dialysis tubing (Molecular weight cut of 12000) and membrane filter of pore size 0.22 μm were purchased from Himedia Lab, Mumbai, India. All other chemicals used were obtained from authentic source and were of Analytical Reagent grade.

Equipment

- Analytical Weighing Balance (ATX 224, Shimadzu, Japan)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Ultrasonic Bath Sonicator (Ultrasonics Selec, Vetra, Italy)
- UV-Visible Spectrophotometer (UV1800, Shimadzu, Japan)
- Magnetic Stirrer (Remi Instruments, Mumbai, India)
- Cooling centrifuge (Remi Equipments, Mumbai, India)
- Malvern Zetasizer (NanoZS, Malvern Instruments, UK)
- Transmission Electron Microscope Tecnai 20 (Philips, Holland)
- Scanning Electron Microscope XL 30 ESEM (Philips, Netherlands)

6.3 Methods

6.3.1 Vesicle Size

The Vesicle size of all the three optimized formulations were determined using Dynamic light scattering using Malvern Zetasizer (NanoZS, Malvern Instruments, UK) by the method already described in chapter 5. All the experiments were performed in triplicate.

6.3.2 Zeta Potential

The Zeta Potential of all the three optimized formulations was determined using Dynamic Light Scattering using Malvern Zetasizer (NanoZS, Malvern Instruments, UK). 50 µL of liposomal dispersion was added to 2 ml distilled water. Each diluted sample was placed in a small disposable zeta cell and the zeta potential was recorded. [1] Care was taken to avoid air bubble entrapment in the zeta cell to ensure the correct measurements. All the experiments were performed in triplicate.

6.3.3 % Entrapment efficiency (EE)

% EE of the all the three optimized formulations was determined using the method described in chapter 5. All the experiments were performed in triplicate.

6.3.4 Drug Loading (% w/w)

Drug Loading (% w/w) was determined for all the three optimized formulations was determined using following method:

The optimized liposomal dispersions were taken in eppendorf tubes and centrifuged (Remi Laboratory Instruments, Mumbai, India) at 20,000 rpm at 4 ^{o}C for 30 minutes. The liposomal pellet settles down while free drug remains in supernatant. The liposomal pellet was air-dried and lysed using methanol. The contents were appropriately diluted and analyzed using UV spectrophotometry at 287 nm for Raloxifene HCl and 279 nm for Leuprolide acetate. Following formula was used to calculate the % Loading (w/w).

$$\%\ \text{Loading}\ \left(\frac{\text{w}}{\text{w}}\right) = \frac{\text{Estimated Entrapped drug in liposomes}}{\text{Total weight of formulation}} \times 100$$

To confirm the mass balance, free drug present in supernatant was analyzed by UV-Spectrophotometry. 0.1 ml of supernatant was taken and diluted appropriately and absorbance of resultant solution was measured at 287 nm for Raloxifene HCl and 279 nm for Leuprolide acetate.

6.3.5 Morphological Analysis by TEM and SEM

6.3.5.1 Transmission Electron Microscopy (TEM)

Morphology and structure of the liposomes were studied using Transmission Electron Microscopy Tecnai 20 (Philips, Holland; facility at SICART, Gujarat, India) operating at 200 kV and capable of point-to-point resolution. In order to perform the TEM observations, samples were prepared by applying a drop of the dispersion to a carbon-coated copper grid and left for a minute to allow some of the vesicles to adhere onto the carbon substrate. After removing the excess dispersion with a piece of filter paper, a drop of 1 % Uranyl acetate solution was applied for one minute and then left to be air-dried. The sample was then observed under Transmission Electron Microscope after drying. The magnification power was kept at 25000x-75000x.

6.3.5.2 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy was carried out to study the morphological characteristics (shape and surface) of the different liposomal formulations prepared. The samples for the SEM analysis were prepared by placing a drop of the liposomal dispersion on one side of adhesive stub. The samples were then air dried before performing microscopy. Then they were examined photographically by a scanning electron microscope (XL 30 ESEM; Philips, Netherlands). Magnification of 7,500-20,000 X was used while taking these images.

6.3.6 In Vitro Drug Release study

In Vitro drug release study was done for optimized batch of all the three liposomal formulations. The method followed for all the three formulations was the same.

Liposomal dispersion was filled in dialysis bag (Molecular Cutoff 12000; Himedia Lab, Mumbai, India) with both the ends closed using the closure clips. It was then immersed in 150 ml of simulated vaginal fluid pH 4.5 containing 0.1 % Tween 80 to maintain sink conditions. The medium was maintained at 37±0.5 ^{o}C and 100 rpm speed was kept constant. 1 ml sample was withdrawn at pre-determined time intervals of 1, 2, 4, 6, 8 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 hours or until 100% drug release was obtained. Equal volume of fresh medium was replaced after each sample withdrawn. The withdrawn samples were analyzed using UV spectrophotometry at 287 nm for Raloxifene HCl and 279 nm for Leuprolide acetate. In vitro release study for plain drugs was also performed to compare the results with the liposomal formulations. The results of drug release study were fitted to various mathematical models to evaluate the kinetics of release.

In vitro drug release from Intra Vaginal Rod (IVR) inserts of all the three liposomal formulations was performed in similar manner as described above. The IVR containing freeze-dried rod of liposomal formulation was placed in the diffusion medium (SVF pH 4.5) maintained at 37±0.5 ^{o}C and 100 rpm speed was kept constant. 1 ml sample was withdrawn at pre-determined time intervals of 1, 2, 4, 6, 8 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 hours or until 100 % drug release was obtained. Equal volume of fresh medium was replaced after each sample withdrawn. The withdrawn samples were analyzed using UV spectrophotometry at 287 nm for Raloxifene HCl and 279 nm for Leuprolide acetate.

6.4 Results and Discussion

6.4.1 Vesicle size

The vesicle size of optimized RLX-Liposomes was found to be 122.1±1.2 nm with PDI of 0.327. The vesicle size of optimized LA-Liposomes was found to be 432.5±2.1 nm with PDI of 0.380 while the vesicle size of optimized dual drug loaded (RLX-LA) liposomes was found to be 354.4±1.0 nm with PDI of 0.533. The developed liposomal formulations were homogenous as seen from the PDI values and of desired size range. The larger vesicle size in the case of LA-Liposomes and dual drug loaded (RLX-LA) Liposomes, is due to the dehydration-rehydration method, which yields Multilamellar

vesicles (MLVs). [2] Figure 6.1, 6.2 and 6.3 shows the size distribution plot obtained through Malvern Zetasizer of optimized RLX-Liposomes, optimized LA-Liposomes and optimized dual drug loaded (RLX-LA) Liposomes respectively.

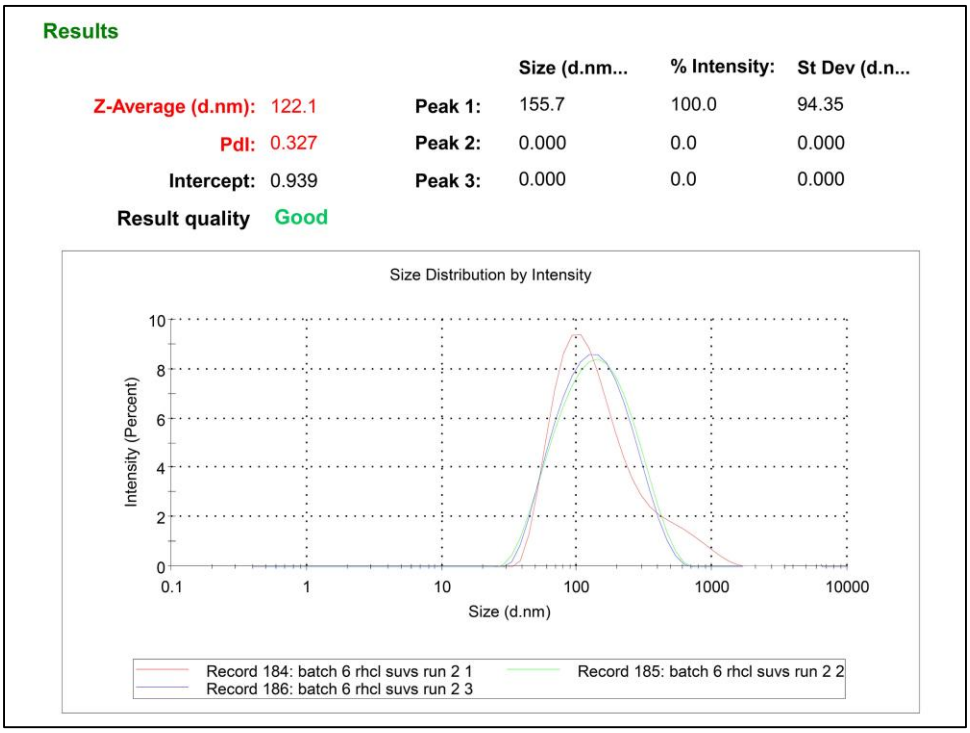

Figure 6.1 Vesicle size of optimized batch of RLX-Liposomes (n=3)

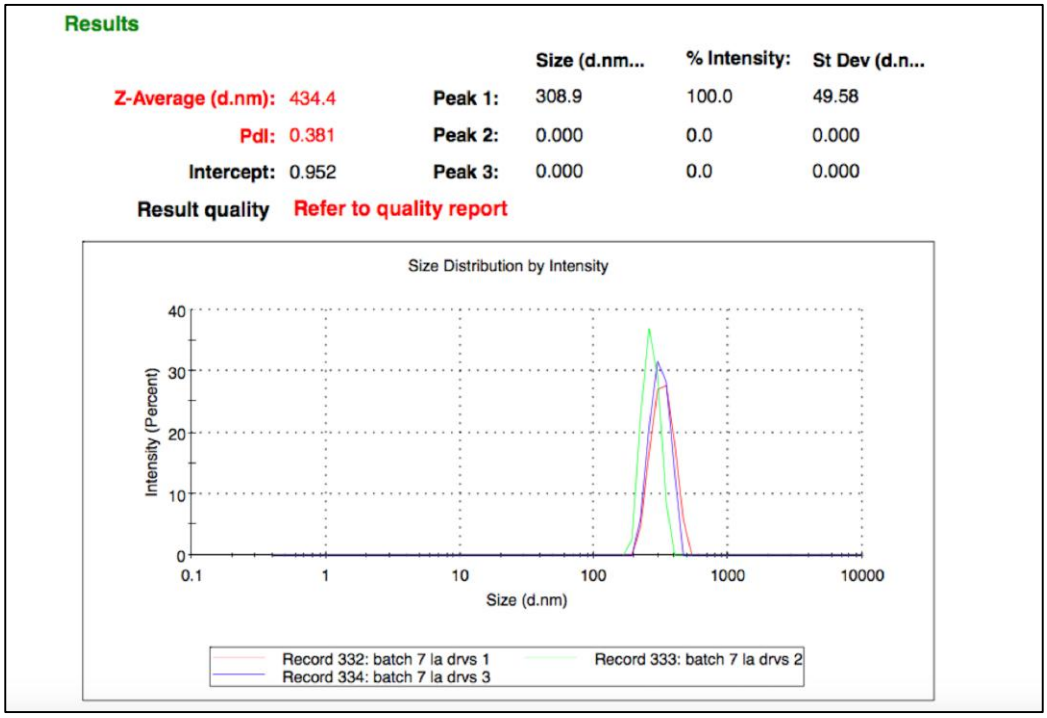

Figure 6.2 Vesicle size of optimized batch of LA-Liposomes (n=3)

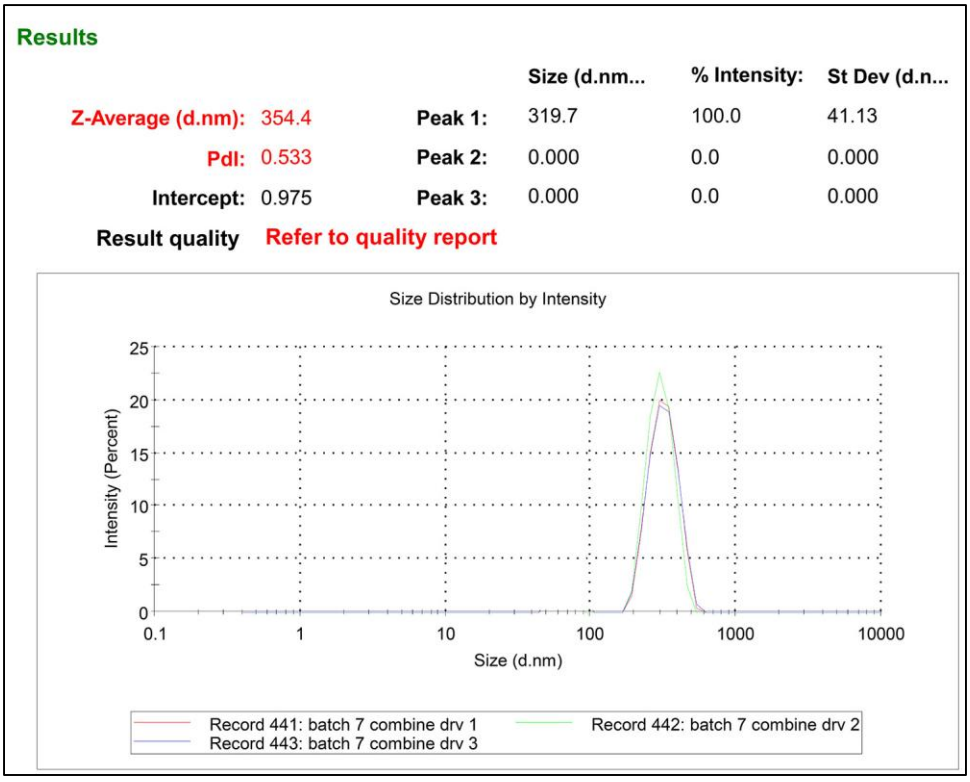

Figure 6.3 Vesicle size of optimized batch of dual drug loaded liposomes (n=3)

6.4.2 Zeta Potential

Zeta potential value for optimized RLX loaded liposomes was +2.13±1.0 mV. For LA loaded liposomes, the optimized batch was having the zeta potential of -23.3±1.1 mV. While in the case of dual drug loaded liposomes, the potential of optimized batch was -22.9±1.2 mV.

Zeta potential measures the effect of electrostatic charges. This is the basic force that causes the repulsion between adjacent vesicles in the liposomal dispersion. Net results are attraction or repulsion depending upon the magnitude of both forces. [3] High absolute value of zeta potential indicates high electric charge on the surface of the drug-loaded liposomes, which can cause strong repellent forces among vesicles to prevent aggregation of the liposomes. If the liposomes have low zeta potential values then attractive Van der Waals forces are able to overcome the repulsive electrical double layer forces and the vesicles tends to come closer, aggregate and the formulation becomes unstable. As a rule liposomes with zeta potentials between +30mV and -30mV are considered stable. [3] Based upon these results we can expect the stability of all the three developed liposomal formulations.

Figures 6.4, 6.5 and 6.6 represent the Zeta Potential plots of RLX-liposomes, LA-Liposomes and RLX-LA liposomes obtained from Malvern Zetasizer.

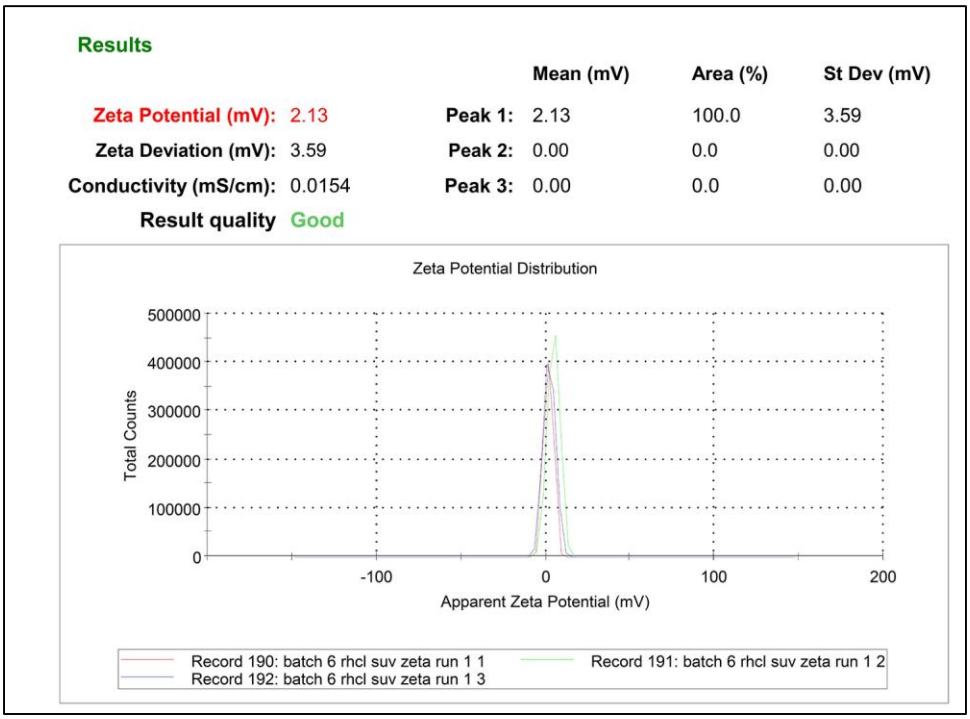

Figure 6.4 Zeta potential of optimized batch of RLX loaded Liposomes

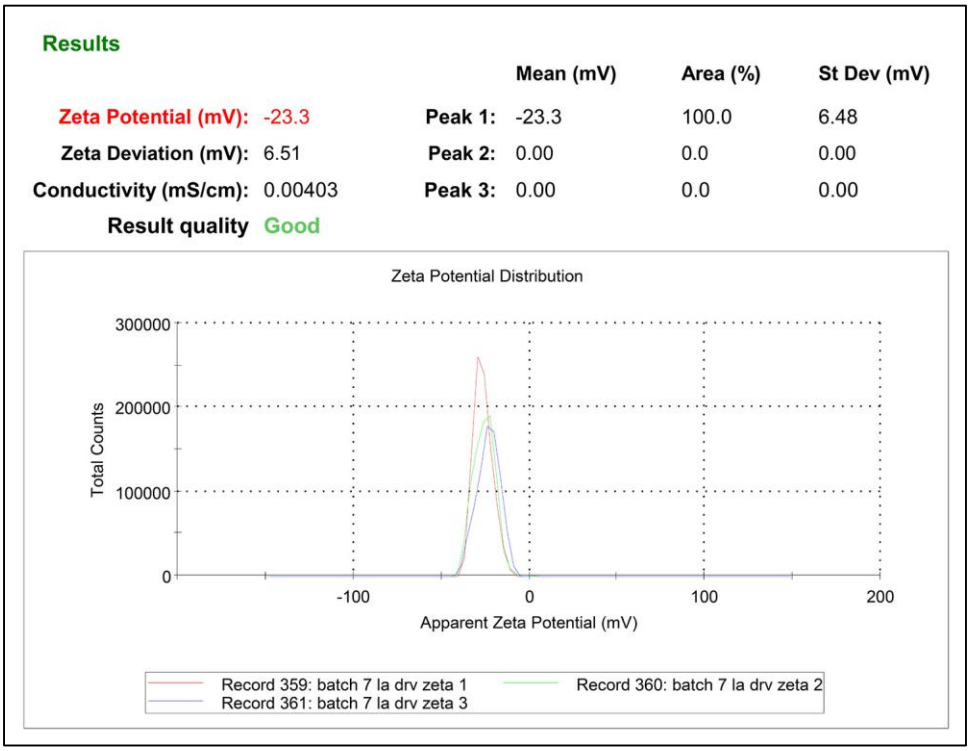

Figure 6.5 Zeta potential of optimized batch of LA loaded Liposomes

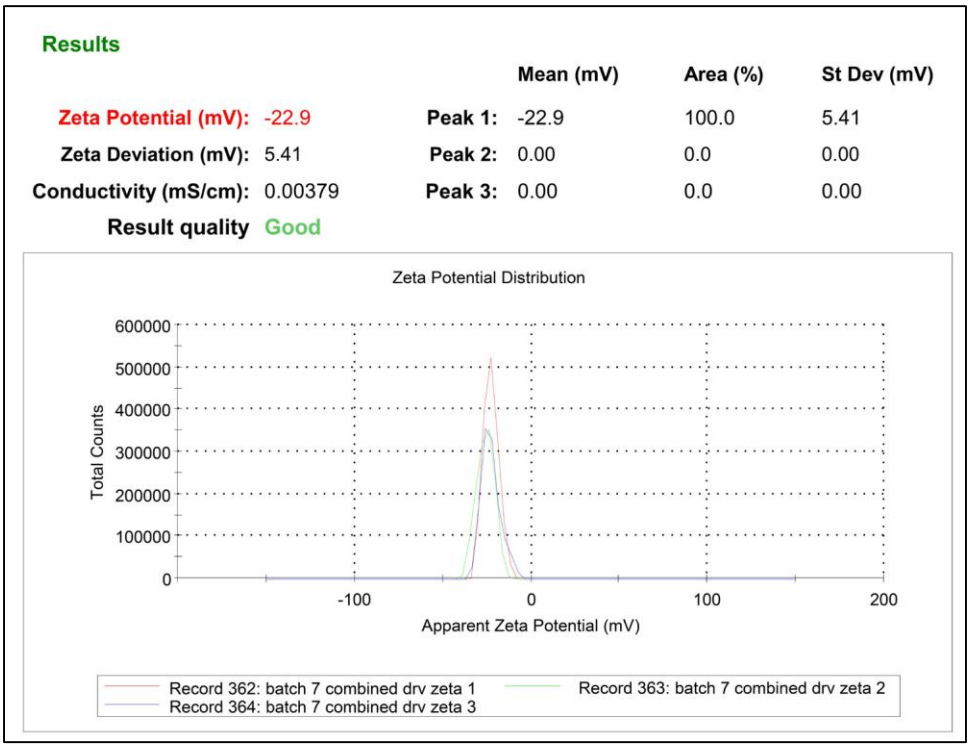

Figure 6.6 Zeta potential of optimized batch of dual drug (RLX-LA) loaded Liposomes

6.4.3 % Entrapment efficiency

The optimized batch of RLX-Liposomes had % EE of 90.96±1.4. While optimized batch of LA-Liposomes had % EE of 74.63±1.1 and optimized batch of dual drug loaded liposomes showed entrapment of RLX to be 90.91±1.1 and of LA to be 74.3±1.0. The reason for high entrapment efficiency obtained for both the drugs is due to the use of DSPC lipid. DSPC with higher phase transition temperature generally exhibit higher stability and is capable of maintaining entrapped substances with less leakage from the vesicles. RLX being a lipophilic drug gets loaded within the lipidic bilayers with a high efficiency. Whereas, for the hydrophilic drug LA, DSPC along with Cholesterol provides a rigid bilayer structure preventing the leakage of the drug from the aqueous core of the vesicles. Also the dehydration-rehydration method results in the formation of MLVs with high entrapment of hydrophilic drugs in the aqueous core. [2, 4, 5]

6.4.4 Drug Loading (% w/w)

The optimized batch of RLX-Liposomes had a loading of 25.29 % w/w and LA-Liposomes had 9.29 % w/w. While in the case of dual drug loaded liposomes, loading of RLX was 18.79 % w/w and that of LA was 7.65 % w/w. % w/w loading value was lower in case of dual drug entrapped liposomes due to increase in total weight of formulation (as both the drugs were added to liposomal formulation).

High drug loading efficiency is due to the use of DSPC as lipid. Rigid liposomes generally exhibit higher stability and are capable of maintaining entrapped substances. Use of longer chain alkyl lipids like DSPC produces more stable liposomes, since the longer alkyl chains, with stronger cohesion, results in less leakage from bilayers and aqueous core of liposomes. The higher drug loading for both the drugs RLX and LA with long alkyl chain DSPC lipid has been attributed to the increased bilayer lipophilic area within systems formed by this longer chain lipid for the hydrophobic drug to accommodate within. [4] RLX being a lipophilic/hydrophobic drug gets loaded within the lipidic bilayers with a high efficiency. Whereas, for the hydrophilic drug LA, DSPC along with Cholesterol provides a rigid bilayer structure preventing the leakage of the drug from the aqueous core of the vesicles. [5] Loading efficiency of both the drugs increased simultaneously with increasing Lipid: Cholesterol ratio in all the three formulations.

6.4.5 Morphological Analysis by TEM and SEM

SEM technique is based on scattered electrons while TEM is based on transmitted electrons. While SEM focuses on the sample's surface and its composition, TEM provides the details about internal composition. Therefore TEM can show many characteristics of the sample, such as morphology, crystallization, stress or even magnetic domains. On the other hand, SEM shows only the morphology of samples. Thus, TEM has much higher resolution power than SEM. However, SEM allows for large amount of sample to be analyzed at a time whereas with TEM only small amount of sample can be analyzed at a time. Therefore, in the present investigation both TEM and SEM were

performed in order to the get the minutest details possible to study the morphology and internal structure about the developed liposomal formulations.

The results of TEM analysis (Figures 6.7, 6.8 and 6.9) showed that all vesicles seemed to have a similar spherical shape. In addition, TEM results were in agreement with size obtained by Dynamic Light Scattering for all the three liposomal formulations. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of liposomal vesicles. The inner areas of the liposomes appeared dark which might be attributed to dense packing of the lipid bilayer vesicles. TEM is a powerful tool for studying surface morphology and lamellarity of the prepared liposomes. Multiple lamellas were visible in the microscopic images for dehydrated-rehydrated vesicles as seen in Figure 6.8 and 6.9.

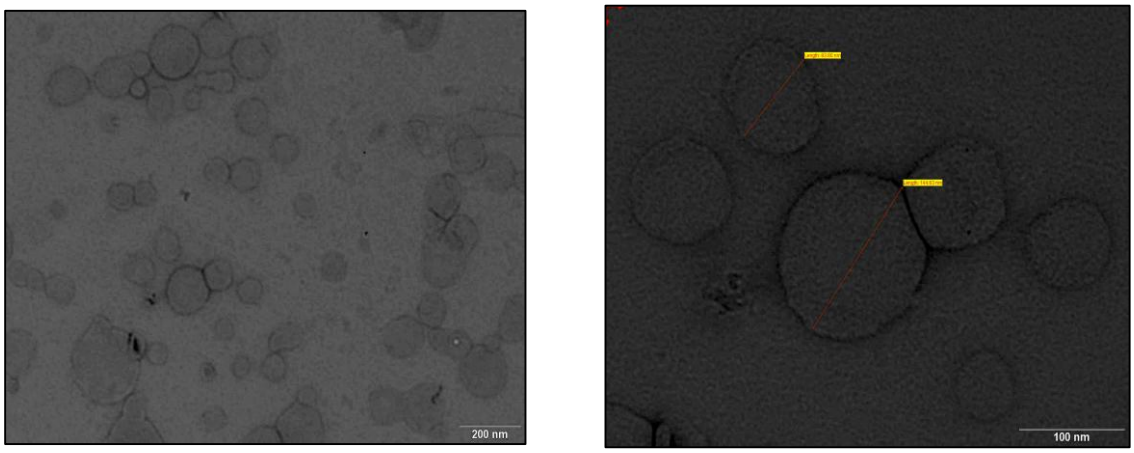

Figure 6.7 TEM images of RLX loaded liposomes

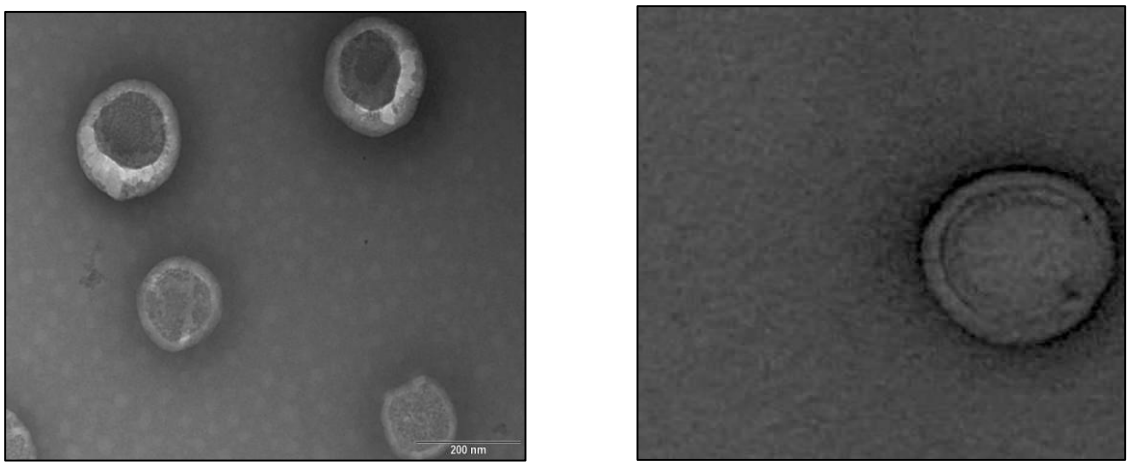

Figure 6.8 TEM image of LA loaded liposomes

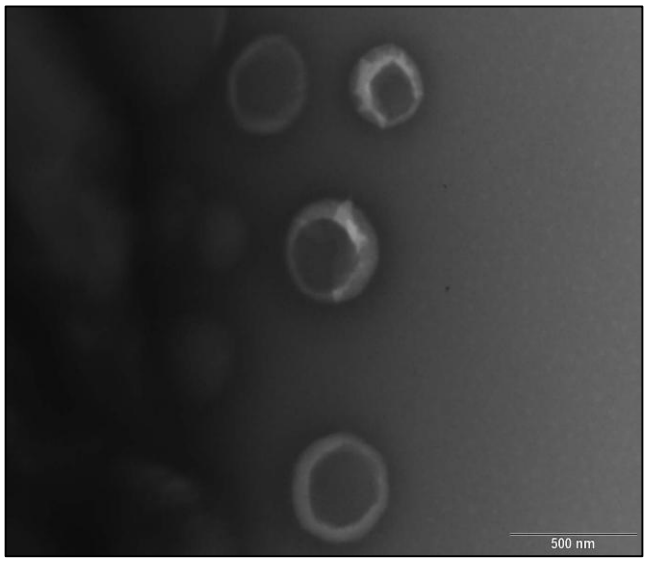

Figure 6.9 TEM image of dual drug (RLX-LA) loaded liposomes

The results of SEM analysis are seen in Figures 6.10, 6.11 and 6.12. The images reveal that the vesicles were discrete, round, uniform in shape with no signs of aggregation.

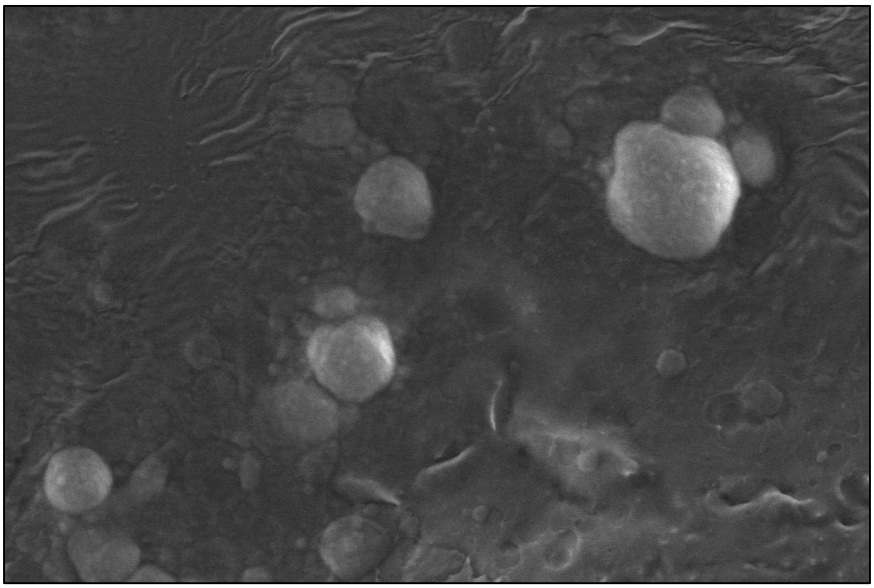

Figure 6.10 SEM image of RLX loaded liposomes

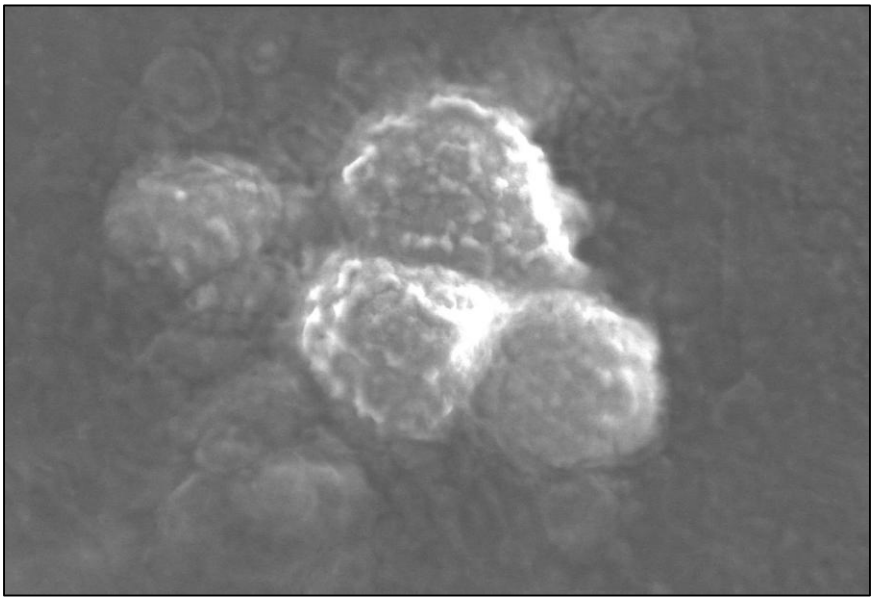

Figure 6.11 SEM image of LA loaded liposomes

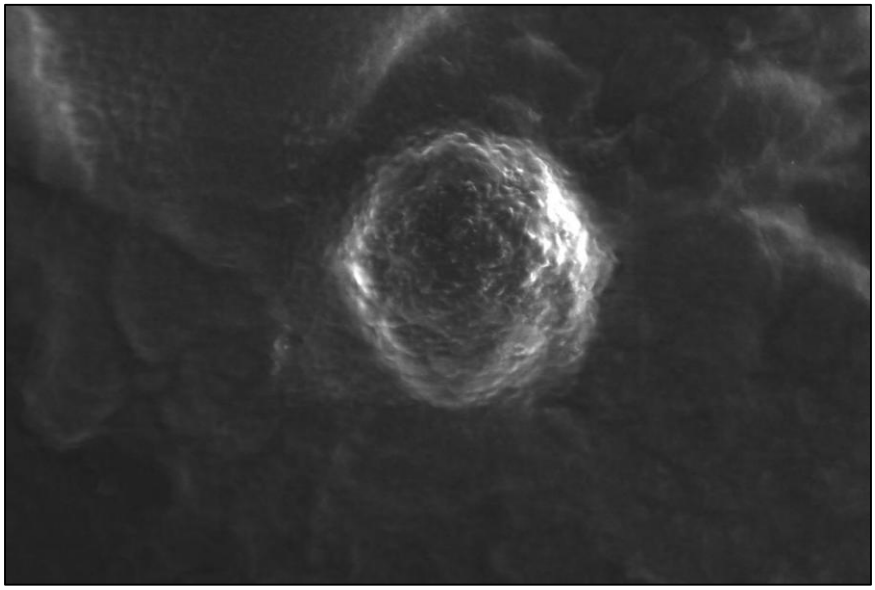

Figure 6.12 SEM image of dual drug (RLX-LA loaded) liposomes

6.4.6 In Vitro Drug Release

6.4.6.1 Drug Release from Liposomal dispersions

In vitro drug release data expressed as % drug released over time determined for optimized formulation of liposomes is shown in Figure 6.13, 6.14 and 6.15 for RLX-liposomes, LA-liposomes and dual drug entrapped liposomes respectively.

RLX loaded liposomes sustained the drug release for up to 132 hours (approx. 6 days). Where as, the entire pure RLX drug was released within 8 hours. In the case of LA loaded liposomes, drug release could be achieved for 108 hours (approx. 5 days). Pure LA was entirely released in the diffusion medium within 4 hours. Similar results were obtained for dual drug entrapped liposomes. There was no significant difference between the drug release rate from the individual formulations and dual drug entrapped formulation. The results have been shown in Table 6.1, 6.2 and 6.3 for RLX-liposomes, LA-liposomes and dual drug entrapped liposomes respectively. RLX release could be sustained for longer time than the LA due to the lipophilic nature of RLX. RLX being able to get entrapped more strongly within the lipidic bilayers of DSPC gets released at

slower rate than the hydrophilic drug LA. Based upon these results, we can expect to deliver the drugs in vivo for a week.

Over all, slower release of drug from liposomes than the plain drugs is attributed to increased diffusional distance and hindrance effect by the surrounding lipidic bilayer. DSPC and Cholesterol gives sustained release of drug for longer periods of time, as worked upon by Judith and Gregoriadis. [6] Incorporating phosphatidylcholine with lower phase transition temperature into the liposomal membrane (i.e., lipids with unsaturated and/or shorter fatty acyl chains) increases drug leakage rates compared to liposomes containing lipids with higher phase transition temperatures. DSPC gives good stability to liposomes in terms of rigidity of the lipid bilayer with a high phase transition temperature, long fatty acid side chains, high degree of unsaturation and polar head groups. [2]

Table 6.1 In Vitro Drug release of RLX loaded optimized liposomes

Time (Hours)	**% Cumulative Drug Release**	
	RLX-Liposomes	**Plain Drug (RLX)**
0	0	0
0.5	5.94±1.1	11.87±1.2
1	6.19±1.2	19.6±1.4
2	6.71±0.89	36.84±2.1
4	7.45±1.0	62.5±1.8
6	8.33±1.4	85.1±1.4
8	9.16±1.1	98.89±0.89
24	10.77±1.0	-
36	14.98±1.3	-
48	23.97±1.6	-
60	34.08±1.2	-
72	42.22±1.0	-
84	57.58±1.4	-
96	68.6±1.5	-
108	76.14±1.7	-
120	85.71±1.8	-
132	98.4±1.1	-

*Experiment was done in triplicate (n=3)

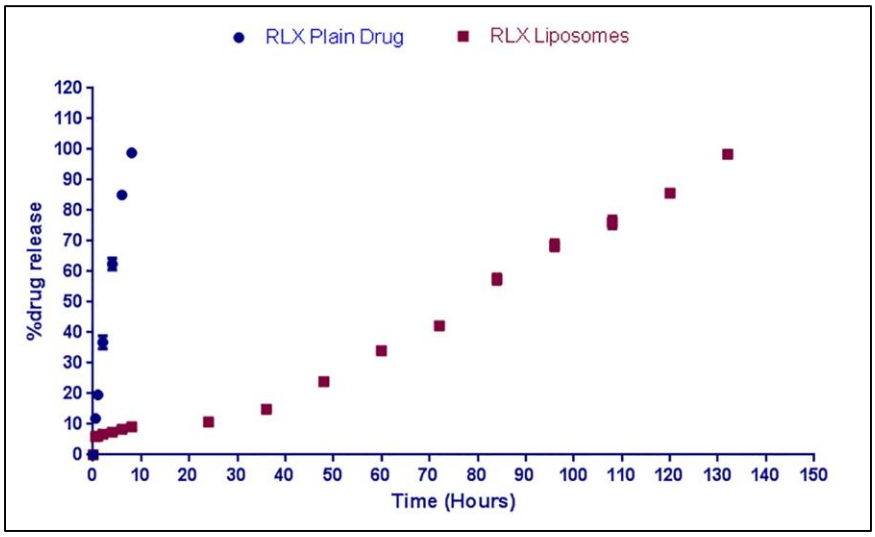

Figure 6.13 In Vitro Drug release profile of RLX loaded optimized liposomes

Table 6.2 In Vitro Drug release of LA loaded optimized liposomes

Time (Hours)	% Cumulative Drug Release	
	LA-Liposomes	Plain Drug (LA)
0	0	0
0.5	8.48±1.1	28.87±1.2
1	10.22±1.2	55.21±1.1
2	11.73±1.4	78.08±1.4
4	12.77±1.2	96.78±1.3
6	14.03±1.1	
8	14.48±1.3	
24	28.21±1.5	-
36	43.35±1.4	-
48	54.80±1.1	-
60	61.50±1.5	-
72	68.21±1.1	-
84	77.08±1.2	-
96	89.93±1.2	-
108	98.55±1.4	-

*Experiment was done in triplicate (n=3)

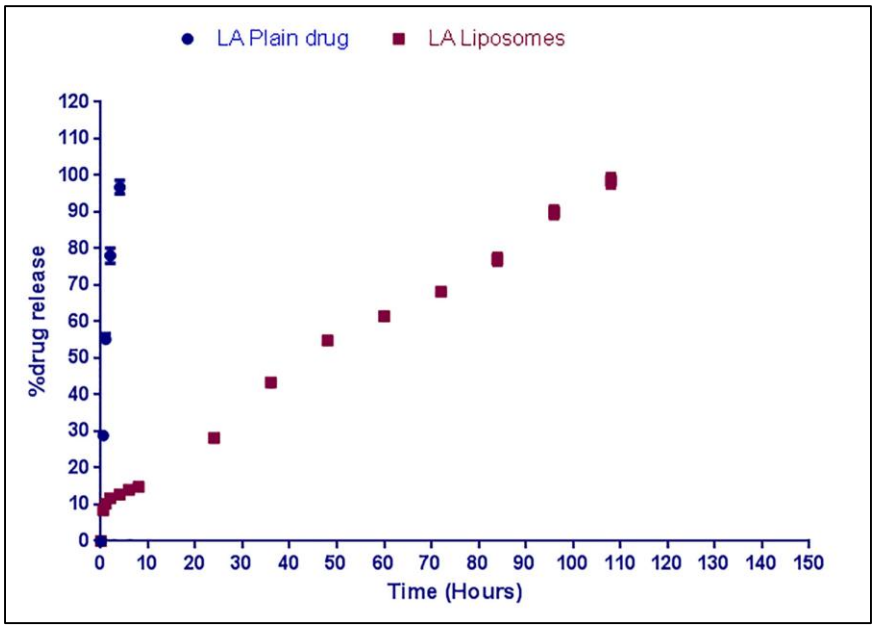

Figure 6.14 In Vitro Drug release profile of LA loaded optimized liposomes

Table 6.3 In Vitro Drug release of Dual Drug loaded optimized liposomes

Time (Hours)	% Cumulative Drug Release	
	RLX	LA
0	0	0
0.5	5.99±1.1	8.74±1.2
1	6.25±1.2	10.48±1.1
2	6.78±1.4	12.06±1.4
4	7.52±1.2	13.15±1.3
6	8.36±1.1	14.37±1.2
8	9.18±1.3	15.04±1.5
24	10.79±1.5	28.57±1.6
36	15.08±1.4	42.78±1.3
48	24.07±1.1	53.95±1.1
60	34.18±1.5	60.68±1.0
72	43.38±1.1	67.42±1.4
84	58.18±1.2	76.47±1.7
96	69.52±1.2	88.79±1.4
108	77.31±1.2	97.64±1.5
120	86.46±1.1	-
132	99.03±1.3	-

*Experiment was done in triplicate (n=3)

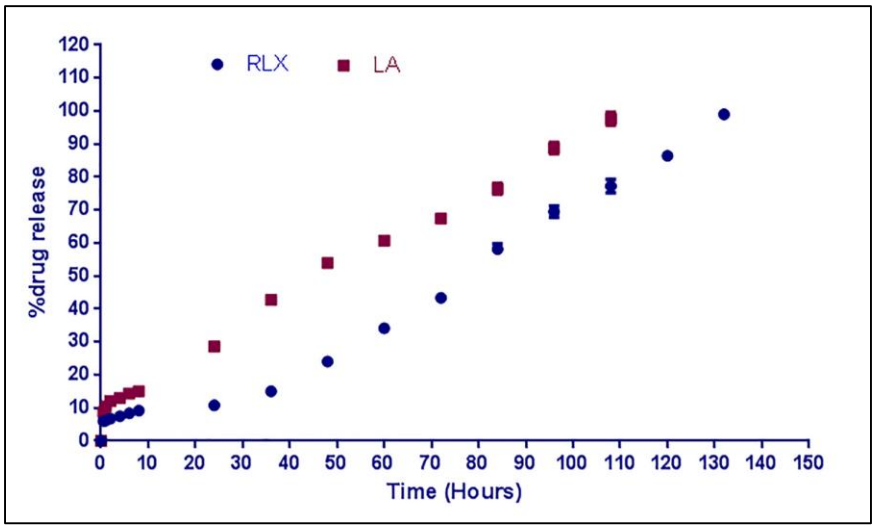

Figure 6.15 In Vitro Drug release profile of Dual Drug loaded optimized liposomes

Drug release kinetics when studied by applying various mathematical models to the drug release data, the release from all the liposomal formulations followed zero order with highest R^2 value of 0.995. It means that the release is independent of the drug concentration. Zero-order release is the mechanism in which a drug is released at a constant rate and is the ultimate goal of all controlled-release drug-delivery systems.

6.4.6.2 Drug Release from Intra Vaginal Rod Inserts (IVRs)

Table 6.4 shows the data for the drug release from the IVR loaded with freeze dried RLX-liposomal dispersion and freeze dried LA-liposomal dispersion. As observed from the data, IVRs sustained the drug release up to 144 hours (6 days) for both RLX as well as LA. Figure 6.16 and 6.17 represents the data graphically.

Table 6.4 In Vitro drug release from liposomal RLX loaded IVR and liposomal LA loaded IVR

Time (Hours)	% Cumulative Drug Release	
	RLX IVR	LA IVR
0	0	0
1	1.85±1.1	4.45±1.1
2	2.67±1.5	5.92±1.3
4	3.14±1.9	6.87±2.1
6	3.62±1.4	7.15±1.4
8	4.13±1.4	7.67±1.5
24	5.54±1.2	8.12±1.8
36	9.95±1.6	10.24±1.2
48	18.06±1.3	20.51±1.3
60	28.98±1.6	31.46±1.7
72	35.52±1.2	38.60±1.6
84	47.15±1.1	51.98±1.2
96	58.94±1.4	60.62±1.3
108	66.34±1.7	71.12±1.1
120	74.14±1.4	79.77±1.1
132	87.09±1.8	91.07±1.1
144	97.27±1.1	99.31±1.2

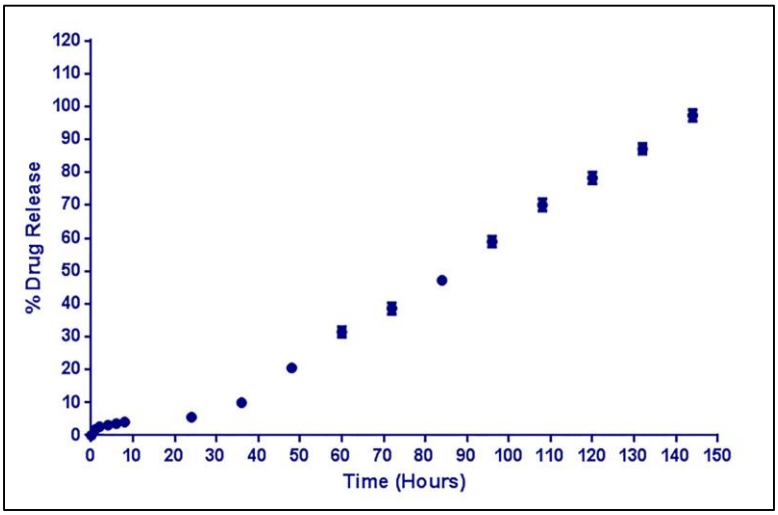

Figure 6.16 In Vitro drug release profile of RLX-liposomes from IVR

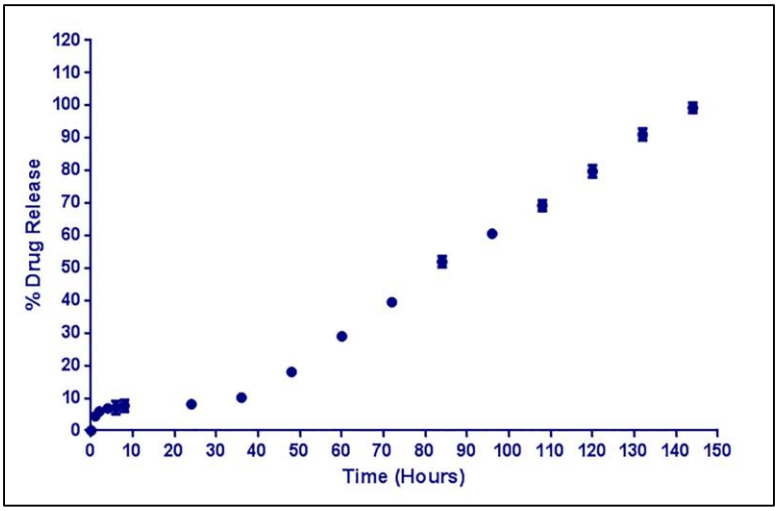

Figure 6.17 In Vitro drug release profile of LA-liposomes from IVR

Table 6.5 shows the release data for IVR loaded with dual drug entrapped liposomes. Figure 6.18 represents the same graphically. There was no significant difference between the drug release profiles of individual liposomal formulation loaded IVR and dual drug entrapped liposomal formulation loaded IVR.

Table 6.5 In Vitro drug release from IVR loaded with dual drug entrapped liposomes

Time (Hours)	**% Cumulative Drug Release from IVR with dual drug entrapped liposomes**	
	RLX	**LA**
0	0	0
1	3.82±1.1	5.9±1.1
2	4.03±1.5	6.63±1.3
4	4.18±1.9	7.24±2.1
6	4.48±1.4	7.94±1.4
8	4.72±1.4	8.4±1.5
24	6.25±1.2	8.98±1.8
36	10.73±1.6	10.82±1.2
48	18.56±1.3	21.11±1.3
60	30.12±1.6	32.08±1.7
72	39.11±1.2	40.28±1.6
84	47.45±1.1	52.98±1.2
96	60.32±1.4	61.45±1.3
108	70.12±1.7	71.25±1.1
120	79.40±1.4	80.91±1.1
132	88.72±1.8	92.06±1.1
144	99.66±1.1	99.98±1.2

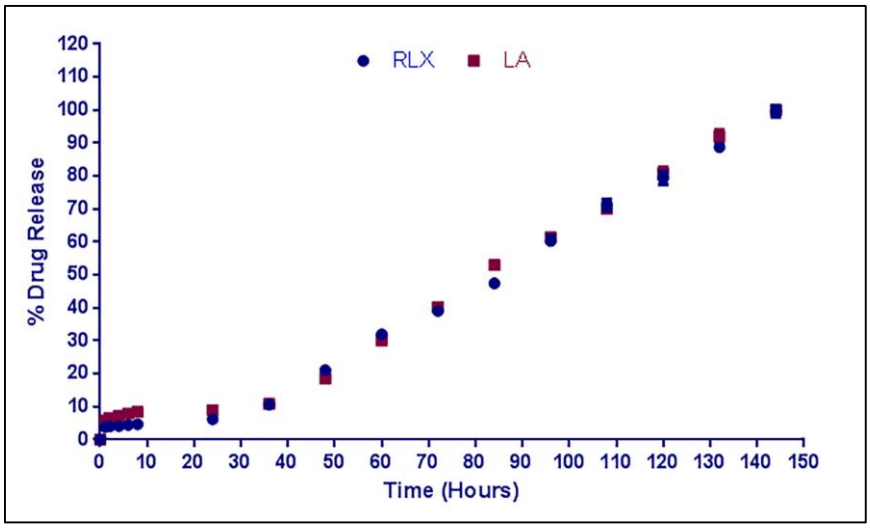

Figure 6.18 In Vitro drug release profile of IVR loaded with dual drug entrapped liposomes

Drug release kinetics when studied by applying various mathematical models to the drug release data, the release was found to follow zero order with highest R^2 value of 0.976. It means that the release is independent of the drug concentration.

6.5 References

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