Chapter 4

PREFORMULATION STUDIES

AND

PRELIMINARY OPTIMIZATION

4.1 Introduction

Preformulation study is defined as an investigation of physical & chemical properties of drug substance alone and when combined with excipients with which it has to be formulated into a complete dosage form. 'A stitch in time saves nine', being the principle behind the Preformulation studies, can prevent the major errors during the product development stage. Preformulation involves the application of bio pharmaceutical principles to the physic-chemical parameters of the drug with the goal of designing an optimum drug delivery system. Characterization of the drug molecule is a very important step at the preformulation phase of product development process. [1]

A formulation scientist utilizes maximum data from preformulation testing to design an appropriate dosage form. Physicochemical properties are used for improving drug solubility, permeability and stability, selecting appropriate excipients and processing conditions to design and evaluate the final dosage form. It has been shown that inadequate preformulation studies is one of the reasons for product recalls. The information required on preformulation will, to a certain extent, depend on the final product, the proposed route of administration and the development plan. [2] Figure 4.1 shows the various preformulation studies that can be performed for a drug candidate.

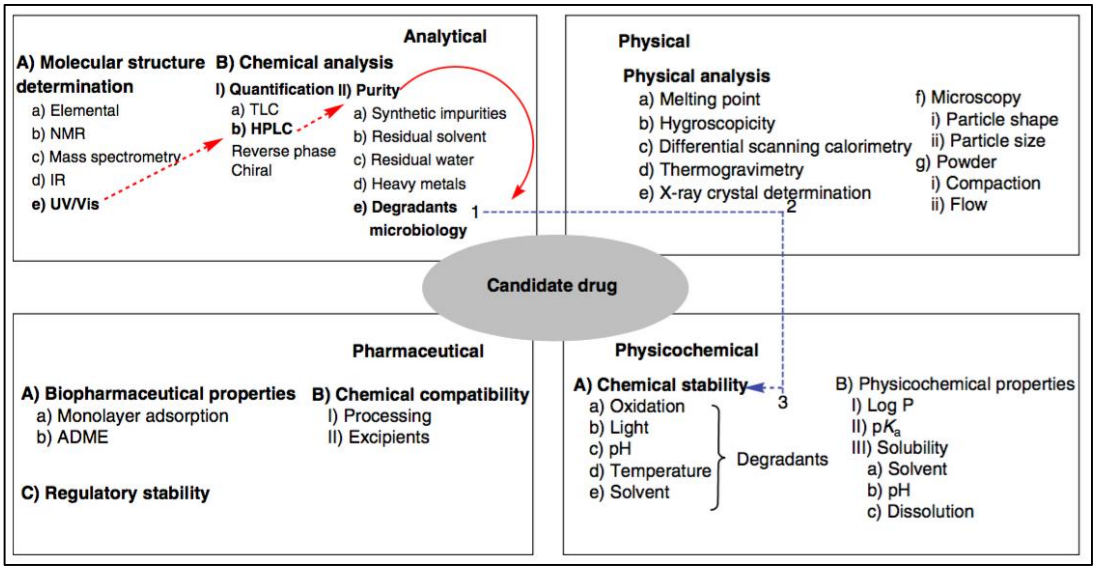

Figure 4.1 Types of Preformulation Studies

4.2 Materials and Equipment

Materials

Leuprolide acetate was obtained as a gift sample from Sun Pharma Advanced Research Centre, Vadodara, India and Raloxifene Hydrochloride was obtained as a gift sample from Aarti drugs Ltd. Mumbai, India. The lipids Hydrogenated Soy Phosphatidylcholine (HSPC), Soy Phosphatidylcholine (SPC) were obtained as a gift sample from Lipoids, Germany. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained as gift sample from Avanti Polar Lipids, Inc. Alabaster, Alabama. Cholesterol was purchased from Sigma Aldrich, Bangalore, India. Dichloromethane, Methanol and Chloroform (A. R. grade) were purchased from Merck, Mumbai, India. Distilled water was prepared using in-house distillation assembly. All other reagents were purchased from S.D. Fine-chemicals limited, Baroda, India 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)
- Differential Scanning Calorimeter (Shimadzu, Japan)
- FTIR (Bruker, Germany)
- Rotary evaporator (IKA RV10, Karnataka, India)
- Particle Size Analyzer 3000 HS (Zeta Sizer Nano Series, Malvern Instruments, UK)
- UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan)
- Probe Sonicator (LabsonicM, Sartorius Ltd, Mumbai, India)
- Cooling Centrifuge (Remi Equipment, Mumbai, India)
- Nikon H600L Microscope (Nikon, Japan)

4.3 Preformulation study

4.3.1 Methods

Identification of Leuprolide acetate and Raloxifene Hydrochloride as well the assessment of drug-excipient compatibility was done by performing following tests:

a) Appearance, Color and Odor: Organoleptic characteristics of the drugs were determined.

b) Melting point: Melting point of the drugs was determined using capillary tube method. Drug was filled in a capillary tube with its one end sealed. This capillary was then tied up with thermometer and immersed in to liquid paraffin bath, which was then heated using a burner. The temperature at which drug starts to melt and when it gets completely melted were noted.

c) Solubility determination: Solubility of the drugs was determined in various solvents by taking minute quantity of drugs (approx. 1-2 mg) in separate pre-labeled test tubes and adding various solvents like distilled water, methanol, dichloromethane and chloroform, drop wise to check by visible observation if the drugs dissolves without leaving any sediments.

d) FTIR Spectroscopy: Obtained sample of drugs were mixed with IR grade anhydrous Potassium bromide in a ratio of 1:100 and pellets were prepared by applying 10 metric ton of pressure in a hydraulic press. The pellets were then scanned over a range of 4000-400cm^{-1} in FTIR instrument (Bruker, Germany). The FT-IR spectra thus obtained was compared with the reference standard FT-IR spectra. Any difference in the principle peaks will judge the purity of the drug samples obtained.

e) DSC: It allows the evaluation of possible incompatibilities, because it shows changes in the appearance and shift of melting endotherms and exotherms, and/or variations in the corresponding enthalpies of reaction. The DSC thermogram of the drug samples were recorded over a temperature range of 30°C to 300°C.

f) Drug- Excipient Compatibility: Assessment of possible incompatibilities between an active pharmaceutical ingredient and the excipients was also done. For this mixture of drugs with different excipients to be used in formulation (DSPC and Cholesterol) were subjected to Fourier Transform Infra-Red (IR) spectroscopy (Bruker, Germany) and Differential Scanning Calorimetric (DSC) studies (Shimadzu, Japan). The results were compared with reference standard FTIR spectra and DSC endotherms of pure drugs.

4.3.2 Results and Discussion

The basic purpose of the pre-formulation studies is to provide a rational basis for the formulation approaches, to maximize the chances of success in formulating an acceptable product, and to ultimately provide a basis for optimizing drug product quality and performance.

4.3.2.1 Results of Preformulation study of Leuprolide acetate

a) Leuprolide acetate, which confirms with its description in US Pharmacopoeia, is a fine or fluffy, white to off-white powder without any odor.

b) The Melting point of Leuprolide acetate was found to be in the range of 169-174°C which complied with the reported value of 170°C. [3]

c) Leuprolide acetate was found to be freely soluble in water with 1mg drug requiring less than 2 ml of water. It was slightly soluble in methanol and chloroform, while it was not soluble in Dichloromethane. It is a hydrophilic drug with reported water solubility of 0.50 mg/ml.

d) The FTIR Spectrum of Leuprolide acetate showed characteristic peaks at 3176 cm^{-1} (due to phenolic –OH), 1650 cm^{-1} (due to C=C stretch), 1251cm^{-1} (due to C-O), 1401 cm^{-1} (due to O-H bend), 2959 cm^{-1} (due to C-H stretch) which complied with its molecular structure and reference FTIR spectrum. This indicated that the obtained sample of drug was pure with no impurities or adulteration. Figure 4.2 shows the FTIR spectrum of Leuprolide acetate having all the principal peaks.

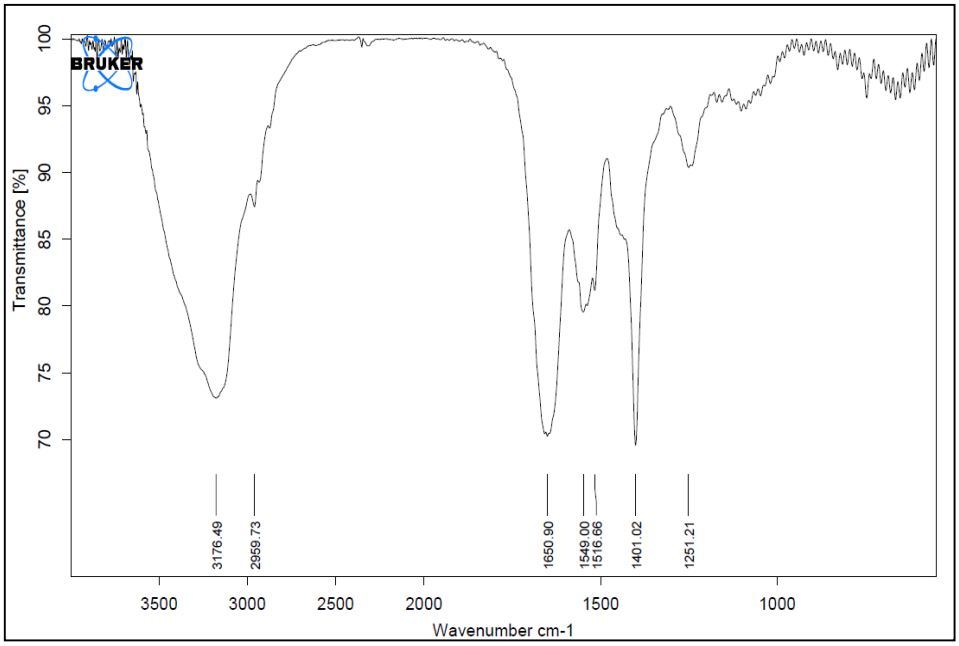

Figure 4.2 FTIR spectrum of Leuprolide acetate

e) DSC: The results of DSC study as shown in Figure 4.3 reveals the endothermic peak of Leuprolide acetate at 170.5°C. The sharp characteristic peak of LA at its melting point and the absence of any other peaks indicates that the drug was pure.

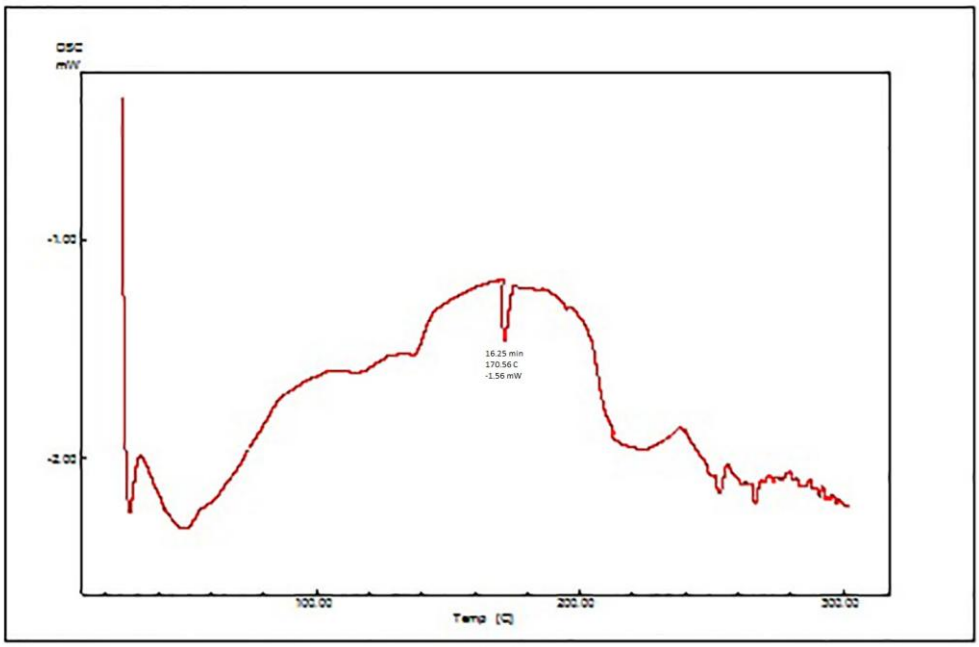

Figure 4.3 DSC thermogram of Leuprolide acetate

f) Drug-excipient compatibility study: The possible interaction between the drug and the excipients was studied by the Fourier transform infrared (FTIR) spectroscopy and DSC analysis. The FTIR results as seen in Figure 4.4 shows that the characteristic peaks of LA were retained. This indicated that there is no chemical interaction between the drug and other excipients (DSPC and Cholesterol) and hence no incompatibility. The summary of peaks is given in Table 4.1

Table 4.1 FTIR peaks of LA and LA-Excipients mixture

Principal peaks of LA.	**Peaks of Drug-Excipient mixture**	**Functional groups**
3176 cm^{-1}	3175 cm^{-1}	Phenolic-OH
1650 cm^{-1}	1643 cm^{-1}	C=C stretch
1251 cm^{-1}	1246 cm^{-1}	C-O
1401 cm^{-1}	1400 cm^{-1}	O-H bend
2959 cm^{-1}	2957 cm^{-1}	C-H stretch

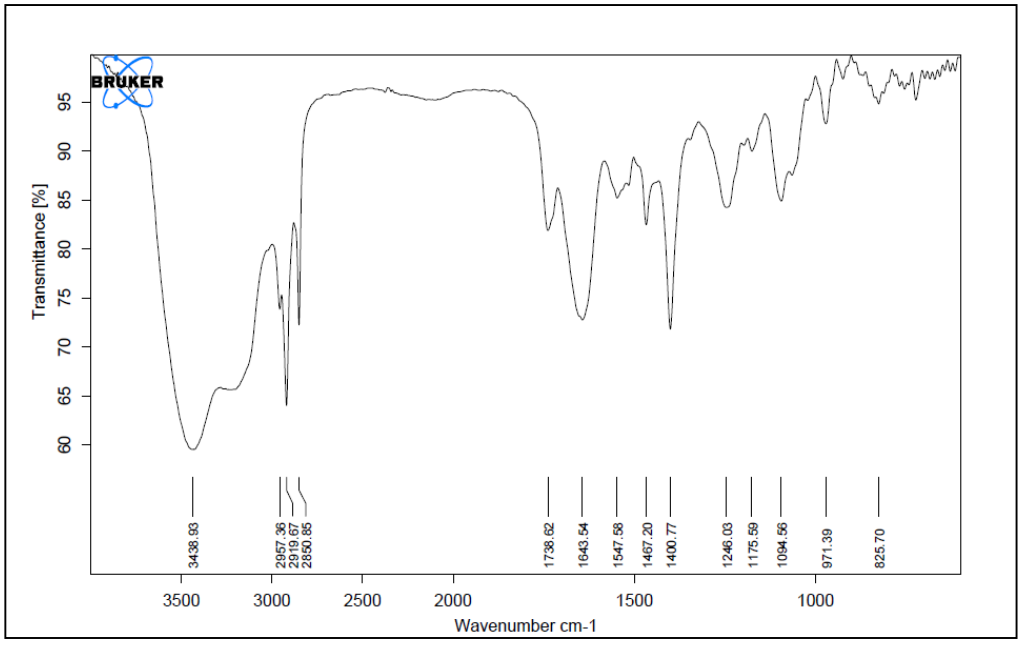

Figure 4.4 FTIR spectrum of LA along with excipients

Similarly, results of DSC analysis showed that the characteristic endothermic peak of LA was retained at its melting point (172.21°C), when analyzed along with excipients,

indicating the drug-excipient compatibility. Figure 4.5 shows the endotherm of LA along with the excipients.

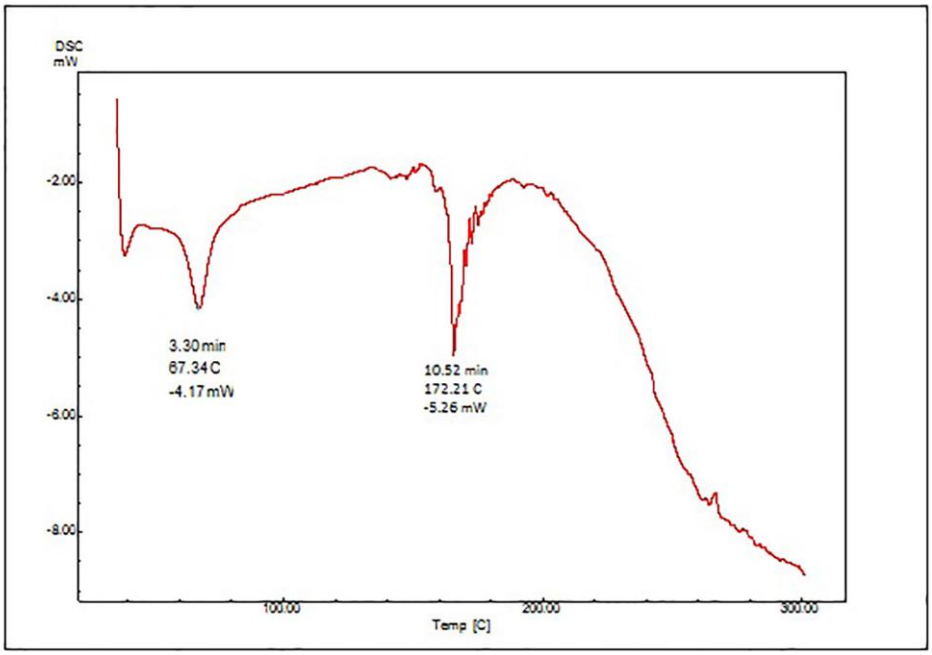

Figure 4.5 DSC thermogram of Leuprolide acetate along with the excipients

4.3.2.2 Results of Preformulation study of Raloxifene Hydrochloride

a) Raloxifene Hydrochloride, which confirms with description in USP 38 NF 33 is off-white to light yellow amorphous powder without any odor.

b) The Melting point of Raloxifene Hydrochloride was found to be in the range of 265-270°C, which complied with the reported value of 267°C. [4]

c) Raloxifene Hydrochloride was found to be freely soluble in methanol with 2 mg drug requiring less than 1 ml of methanol. It was fairly soluble in chloroform and dichloromethane, while it was insoluble in water. It is a lipophilic drug with reported water solubility of 0.000512 mg/ml.

d) The FTIR Spectrum of Raloxifene Hydrochloride showed characteristic peaks at 3140 cm^{-1}, 1642 cm^{-1} (due to C=O stretching), 1596cm^{-1} (due to C=C stretching), 1258 cm^{-1} (due to C-O stretching), 806 cm^{-1} (due to thiophene C-H) which complied with its

molecular structure and reference FTIR spectrum. This indicated that the obtained sample of drug was pure with no impurities or adulteration. Figure 4.6 shows the FTIR spectrum of Raloxifene Hydrochloride having all the principal peaks.

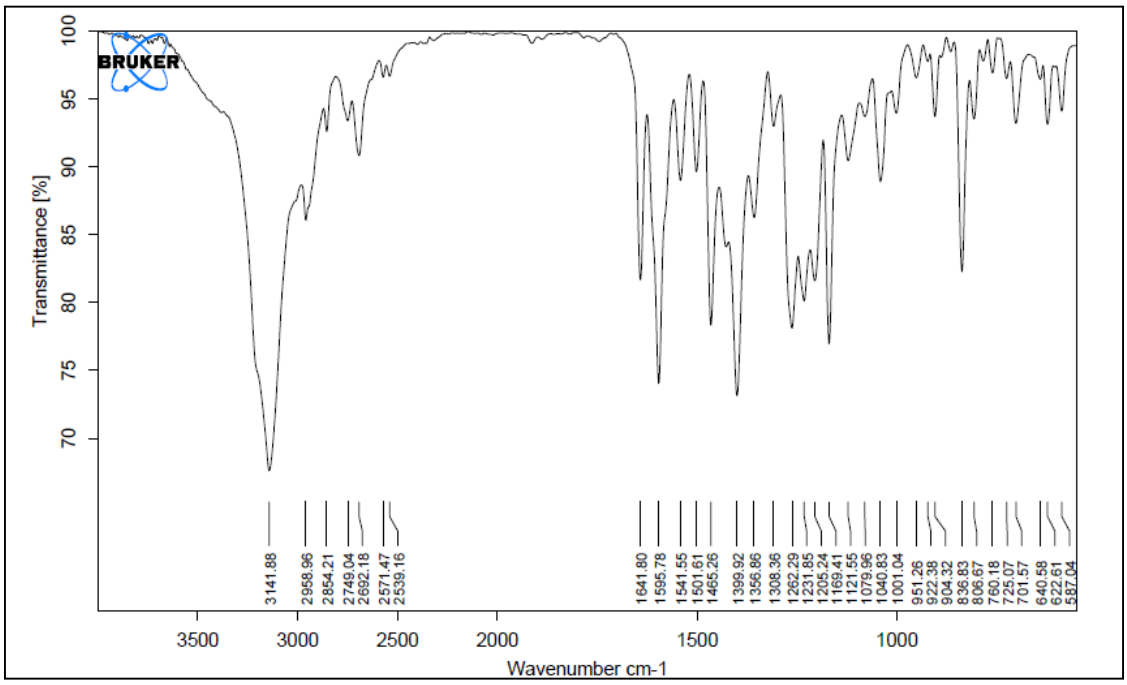

Figure 4.6 FTIR spectrum of RLX

e) DSC: The results of DSC study as shown in Figure 4.7 reveals the endothermic peak of Raloxifene Hydrochloride at 267°C. The sharp characteristic peak of RLX at its melting point indicates that the drug was pure.

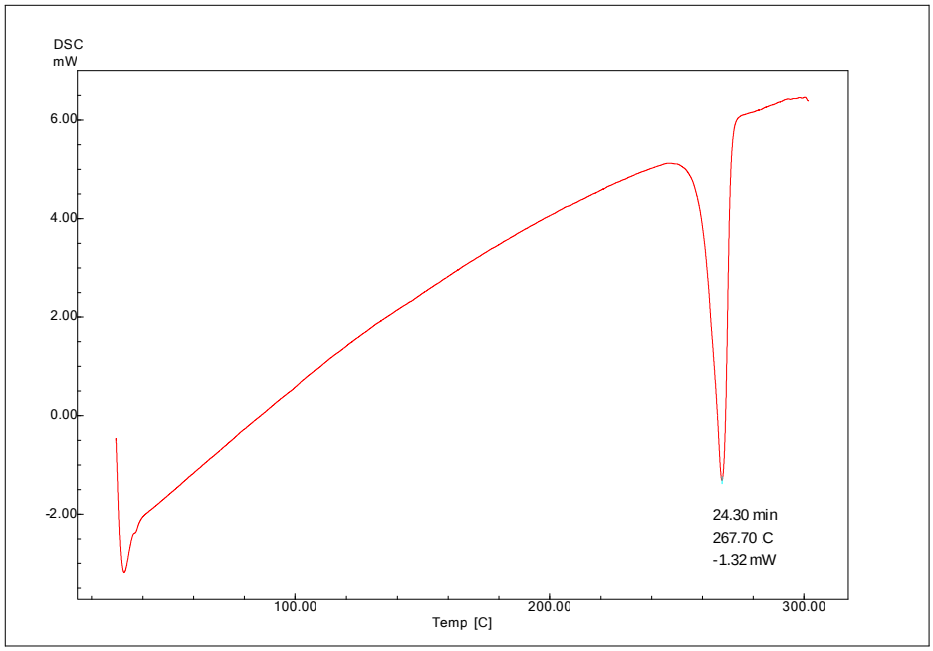

Figure 4.7 DSC thermogram of Raloxifene Hydrochloride

f) Drug-excipient compatibility study: The possible interaction between the drug and the excipients (DSPC and Cholesterol) was studied by the Fourier transform infrared (FTIR) spectroscopy and DSC analysis. The FTIR results as seen in Figure 4.8 shows that the characteristic peaks of RLX were retained. This indicated that there is no chemical interaction between the drug and other excipients and hence no incompatibility. The summary of peaks is given in Table 4.2

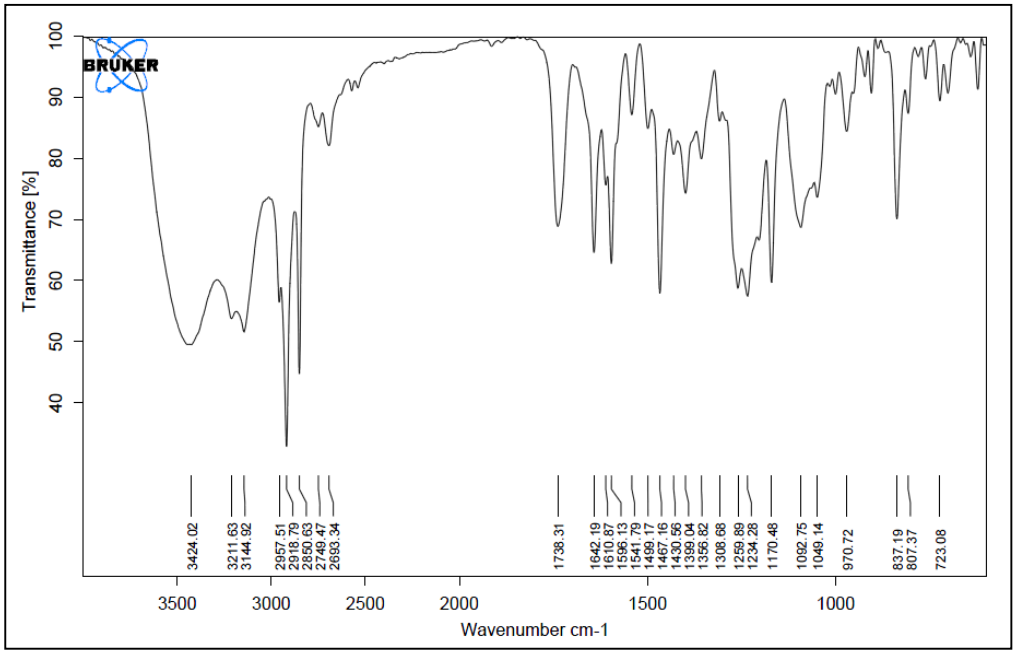

Figure 4.8 FTIR spectrum of RLX along with excipients

Table 4.2 FTIR peaks of RLX and RLX-Excipients mixture

Principal peaks of RLX.	**Peaks of Drug-Excipient mixture**	**Functional groups**
1642 cm^{-1}	1642 cm^{-1}	C=O stretching
1596 cm^{-1}	1596 cm^{-1}	C=C stretching
1258 cm^{-1}	1259 cm^{-1}	C-O stretching
806 cm^{-1}	807 cm^{-1}	due to thiophene C-H

Similarly, results of DSC analysis showed that the characteristic sharp endothermic peak of RLX was retained at its melting point (271°C), when analyzed along with excipients, indicating the drug-excipient compatibility. Figure 4.9 shows the endotherm of RLX along with the excipients.

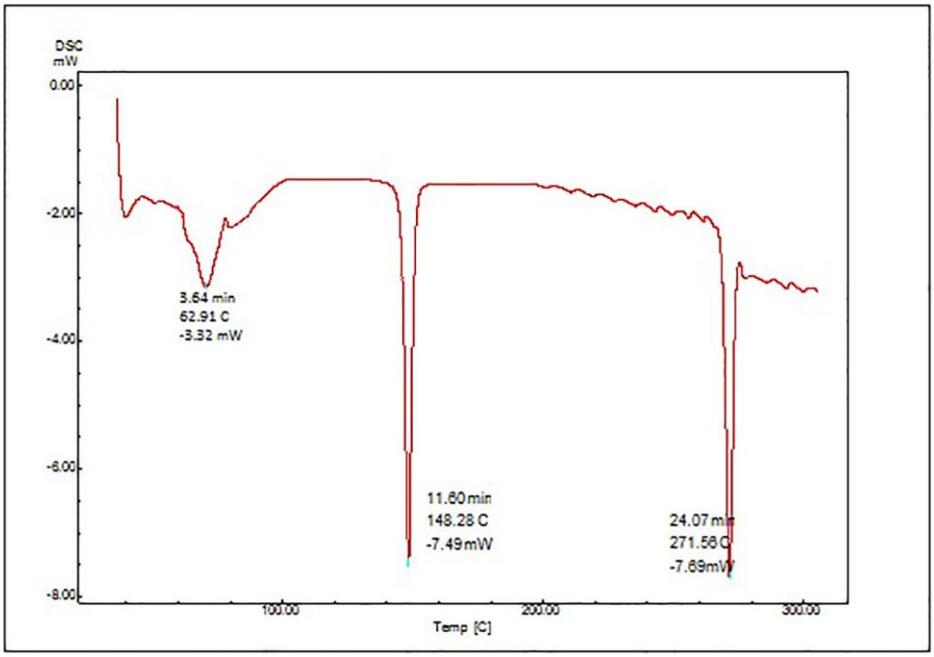

Figure 4.9 DSC thermogram of RLX along with the excipients

4.4 Preliminary Optimization of Formulation Parameters

To start with the formulation development, initially blank liposomes were formulated using Hydrogenated Soya Phosphatidylcholine (HSPC) as lipid by thin film hydration method. Briefly, HSPC: Cholesterol (2:1) was taken in a clean and dry round bottom flask. The mixture was dissolved in methanol: dichloromethane mixture (7:3) (since, HSPC showed maximum solubility in this mixture). The flask was attached to the rotary evaporator (IKA RV10, Karnataka, India) at 60°C. A thin lipid film was thus obtained by evaporation of the organic solvent under vacuum. Then 5 ml of prewarmed (60°C) distilled water was added to the flask as hydration media. The film was allowed to hydrate at 60°C for 1 hour. After hydration, vesicle size of the prepared batch was analysed using particle size analyzer (Zeta Sizer Nano Series, Malvern Instruments, UK). The liposomal dispersion (MLVs) was having the size of 487 nm. This dispersion was then sonicated using probe sonicator (LabsonicM, Sartorius Ltd, Mumbai, India), 3 cycles of 1min each at 70Hz amplitude, to obtain the size of 210 nm. The prepared vesicles are thus the small unilamellar vesicles (SUVs). A drop of dispersion was

observed under the microscope (Nikon H600L Microscope (Nikon, Japan) which confirmed the formation of spherical vesicles as seen in Figure 4.10.

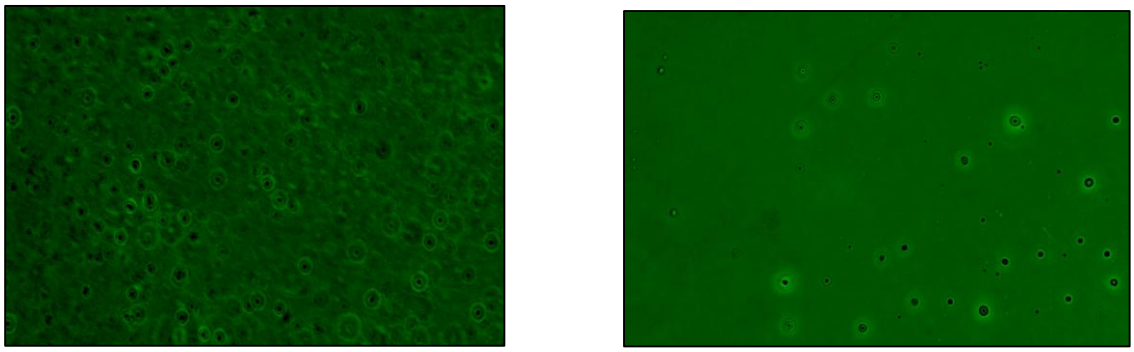

Figure 4.10 Microscopic view of Liposomal vesicles

Liposomes were initially prepared using thin film hydration, ethanol injection method and transmembrane pH gradient method. While thin film hydration method gave high entrapment efficiency for lipophilic Raloxifene Hydrochloride, however, it lead to poor entrapment efficiency and instability of liposomes containing hydrophilic peptide drug Leuprolide acetate leading to aggregate formation and leaching out of drug. Thus the LA-liposomes were then prepared using Reverse Phase Evaporation method. Even this method resulted into poor encapsulation efficiency and poor stability of the prepared formulation. Thus, it was decided to prepare the LA-Liposomal formulation using a modified Dehydration-Rehydration method. In the modified DRV method, preformed empty small unilamellar liposomes are subjected to dehydration, and then the drug destined to entrapment is added to empty liposomes in solution form, resulting in increased entrapment compared to other conventional methods. Hence, Dehydrated Rehydrated Vesicles were prepared which gave better entrapment efficiency with stable liposomes. Also Dehydration-Rehydration is a suitable method for entrapment of peptide-based drugs with high molecular weight. [5] Various trials were also conducted for determination of entrapment efficiency like using sephadex column, using protamine aggregation method and dialysis method. However due to lack of reproducibility of results, high-speed centrifugation method was chosen for further work. Speed chosen was 20000 rpm and time was kept at 30 minutes for complete settlement of liposomal pellet

with clear supernatant.

In preliminary optimization step, the formulation parameters influencing the formation of liposomes were identified and optimized. Various trial experiments were performed based upon which two factors were identified to affect the vesicle size and entrapment efficiency of the liposomes viz. Type of Lipid and Hydration media volume. Therefore these two parameters were optimized in order to formulate the liposomes with desired characteristics.

4.4.1 Type of Lipid

Based upon literature search three lipids i.e. SPC, HSPC, and DSPC were tried for formulating the liposomes loaded with Raloxifene Hydrochloride and Leuprolide acetate. The results of the experiment are shown in Table 4.3, which shows the vesicle size and % entrapment efficiency (% EE) of the liposomal formulations.

Table 4.3 Selection of Lipid in the preliminary optimization of Liposomes

Lipid	**Vesicle Size (nm)**			**% EE**	
	RLX-Liposome	**LA-Liposome**	**RLX-LA Liposome**	**RLX-Liposome**	**LA-Liposome**
SPC	287 ±1.2	593 ±2.3	637 ±2.4	59.40 ±2.9	20.45 ±3.3
HSPC	260 ±2.4	556 ±4.3	564 ±1.6	71.29 ±1.4	39.30 ±2.3
DSPC	**123 ±1.1**	**430 ±1.2**	**358 ±1.2**	**89.98 ±2.1**	**72.2 ±1.3**

*Experiment was done in triplicate (n=3)
Ratio of Lipid: Cholesterol was kept constant as 1:1 for all lipids

Based upon these results, DSPC was chosen as the lipid for the formulation development since, the other two lipids resulted in poor entrapment efficiency with high vesicular size. DSPC has a high transition temperature (56^{o}C) due to long alkyl lipid chain and has the strongest chain-chain interactions resulting in closely packed structure. Longer chain alkyl lipids produce more stable liposomes, since the longer alkyl chains, with stronger cohesion, results in less leakage of drug from the aqueous core of liposomes. [6] Thus, it can also provide enhanced encapsulation efficiency for dual drug entrapment i.e. both lipophilic as well as hydrophilic drug in the liposomes.

4.4.2 Volume of Hydration media

Based upon the literature search distilled water was chosen as the hydration media since it gives the most acceptable range of zeta potential necessary for maintaining the stability of liposomes. However, the volume of water used was found to affect the % EE and the vesicle size (to some extent). The results of the preliminary optimization of volume of hydration media are given in Table 4.4.

Table 4.4 Selection of volume of Hydration media in the preliminary optimization of Liposomes

Volume of water	**Vesicle Size (nm)**			**% EE**	
	RLX-Liposome	**LA-Liposome**	**RLX-LA Liposome**	**RLX-Liposome**	**LA-Liposome**
2 ml	**122 ±1.2**	**432±2.3**	**359 ±1.3**	**89.40 ±2.5**	**73.45 ±1.1**
6 ml	125 ±2.4	437±1.3	361±1.4	78.35 ±2.4	61.26 ±1.3
10 ml	126±1.1	438 ±1.2	363±1.1	72.12 ±3.1	56.15 ±2.1

*Experiment was done in triplicate (n=3)

As seen in the results, as the volume of hydration media was increased, the vesicle size was not affected to a significant level, but the % EE was found to decrease due to increase lipid: aqueous ratio. Hence, 2 ml of the hydration media was chosen for the further optimization of final formulation.

In a nutshell, following parameters were chosen for further optimization of the final dosage form.

- *For Raloxifene Hydrochloride loaded liposomes:*

Lipid: DSPC (1,2-Distearoyl- sn-glycero-3-phosphocholine)
Method: Thin Film Hydration
Hydration Volume: 2 ml

- *For Leuprolide acetate loaded liposomes*

Lipid: DSPC (1,2-Distearoyl- sn-glycero-3-phosphocholine)
Method: Dehydration-Rehydration
Hydration Volume: 2 ml

- *For dual drug loaded liposomes*

Lipid: DSPC (1,2-Distearoyl- sn-glycero-3-phosphocholine)
Method: Dehydration-Rehydration
Hydration Volume: 2 ml

4.5 Preliminary Optimization of Process Parameters

In preliminary optimization step, the process parameters influencing the formation of liposomes were identified and optimized. Among the various parameters that could affect the % EE and vesicle size was speed of rotation of round bottom flask ((RBF) expressed as rotations per minute (RPM)) during lipid film formation and sonication cycle.

4.5.1 Speed of rotation of RBF during lipid film formation

Speed of rotation affects the distribution and homogeneity of the film within the flask [7]. As the solvent is allowed to evaporate under vacuum, the lipids will be deposited as a film within the flask. However, the distribution of the film will be affected by the rotation speed [8]. As a result, the size of liposomes will be affected when the lipid layer is hydrated since a uniform and thin film will help in producing uniform small sized particles. Table 4.5 shows the results of preliminary optimization of speed of rotation of RBF during lipid film formation.

Table 4.5 Selection of speed of rotation of RBF during lipid film formation

Speed of rotation (RPM)	**Vesicle Size (nm)**			**% EE**	
	RLX-Liposome	**LA-Liposome**	**RLX-LA Liposome**	**RLX-Liposome**	**LA-Liposome**
100	143 ±1.4	456±2.2	381 ±1.4	74.40 ±1.5	58.45 ±1.4
120	**121 ±2.4**	**433±1.2**	**363±1.2**	**90.15 ±1.4**	**72.56 ±1.1**
140	137 ±1.1	460 ±1.1	378±1.5	77.22 ±2.1	61.66 ±1.2

*Experiment was done in triplicate (n=3)

The results explains that the optimum speed of rotation of RBF was 120 RPM, since the speed above and below that, significantly affect the % EE and vesicle size due to uneven distribution of the lipid film over the RBF.

4.5.2 Sonication cycle

Sonication is most widely used method to convert MLVs into SUVs. The prepared MLVs were subjected to sonication by using probe sonicator. Sonication of RLX-liposomal suspension was optimized by altering time and amplitude to achieve maximum % EE with lowest possible size.

Table 4.6 Selection of sonication cycle in preliminary optimization

Time (min)	Cycle	Size (nm)	% EE
1	50 %, 0.6 x3	156±2.2	91.10±1.1
2	50 %, 0.6 x3	141±2.5	91.22±1.4
3	50 %, 0.6 x3	134±1.8	90.15±1.2
1	**60 %, 0.6 x3**	**122±2.3**	**89.85±2.1**
2	60 %, 0.6 x3	115±1.7	81.32±1.3
3	60 %, 0.6 x3	109±2.4	76.12±2.2
1	70 %, 0.6 x3	95±2.6	68.30±1.7
2	70 %, 0.6 x3	133±2.4	60.24±2.3
3	70 %, 0.6 x3	158±2.4	55.76±1.5

Sonication represents amplitude in % for exposure of 0.6 sec x 3 cycles
*Experiment was done in triplicate (n=3)

The effect of sonication on RLX-liposomal formulation is shown in Table 4.6. As seen in the results, 60 % amplitude at 0.6 s exposure for 3 cycles of one minute gave the desired liposomal formulation with high entrapment efficiency with lowest possible vesicle size. Increasing the time of sonication from one minute to two and three minutes though gave lower vesicle size, but it compromised the entrapment efficiency. Similar effect was found by increasing the amplitude to 70%, the entrapment efficiency was found to decrease while vesicle size was found to increase at 2 min and 3 min of sonication. The reason for this could be attributed to “over processing” of the vesicles, which leads to aggregation or fusion of tiny unstable vesicles formed during sonication, which in turn increases the size of the vesicles. Attractive Vander Waals forces overcome the repulsive potential barrier due to over sonication of the vesicles. [9]

Where as, lower amplitude (50%) produced vesicles with larger size as it was not the sufficient energy to break MLVs to SUVs. There was no significant difference between the entrapment efficiency by keeping the amplitude 50 % and 60 %. Thus, looking at this data, 60 % amplitude at 0.6 s exposure for 3 cycles of one minute was kept as optimized sonication cycle for size reduction of RLX-Liposomes.

4.6 References

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