Preparation, Characterization and Evaluation of Raloxifene Hydrochloride Nanoparticles

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ABSTRACT – RLF ,NP was prepared using PFG-6000. NPs, collected after freeze drying of the nanosuspension prepared by Co-Precipitation, FDNP exhibited increased drug release compared to the pure drug; Before that RLF were selected as drugs for the present study based on poor solubility and low bioavailability. RLF was having 20 % oral bioavailability. After selection of drug were authenticated and passed through several preformulation studies as solubility, morphology, crystallinity, melting point analysis. The morphological evaluation of the drugs was carried out by SEM. The partial crystalline nature of RLF was confirmed from DSC studies.UV & FTIR was conducted for confirming of drug. Kneading method was carried out (1:5) is optimized. Nanosuspension was prepared by using PEG 6000(30%)as stabilizer. The nanosuspensions prepared with PEG-6000 were freeze dried and NPs were collected. These NPs were screened for morphology, crystallinity, drug released, solubility.

   The NPs revealed highest solubility and faster drug released pattern compared to the pure drug. The NPs collected from the nanosuspensions prepared with PEG 6000 illustrate retention of partial crystalline nature of drug; however, the FDNPs exhibited conversion of the drug to a complete crystal These results were confirmed from DSC study.

Keywords- Nanoprticles, Nanosuspension, Freezedried Nanoparticles, Kneading method, Freezedrying.

I. INTRODUCTION

Nanoparticulate Drug Delivery System

Drug delivery systems had an enormous impact on medical technology, greatly improving the performance of many existing drugs and enabling the use of entirely new therapies. Nanoparticles are small colloidal particles which are made of non- biodegradable and biodegradable polymers. Nanoparticles can be defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm. Nanotechnology and nanotechnology-enabled product development is a rapidly growing new frontier, with increasing industrial demand for smaller and smaller particle sizes.[1] The smaller particle sizes may translate to easier particle manipulation and may be instrumental for the creation of advanced materials. In the pharmaceutical and biotechnology industry in particular, nano-engineering has made its mark and is influencing every segment and subspecialty. Particle size reduction offers a significant opportunity for formulators to solve the product development hurdles inherent with poorly water-soluble active pharmaceutical ingredients (APIs).

Approximately more than 90% of all medicines have active ingredient in the form of solid particles. With the development in nanotechnology, it is now possible to produce drug nanoparticles that can be utilized in a variety of innovative ways. New drug delivery pathways can now be used that can increase drug efficacy and reduce side effects. For example, in 2005, the U.S. Food and Drug Administration approved intravenously administered 130 nm albumin nanoparticles loaded with paclitaxel for cancer therapy, which epitomized the new products anticipated based on nanoparticulate systems.[2,3]

Because of the comparable size of the components in the human cells, nanoparticles are of great interest in drug delivery. It appears that nature, in making the biological systems, has extensively used nanometer scale. If one has to go hand in hand with nature in treating the diseases one needs to use the same scale, whether it is correcting a faulty gene, killing leprosy bacteria sitting inside the body cells, blocking the multiplication of viral genome, killing a cancer cell, repairing the cellular metabolism, or preventing wrinkles or other signs of aging. Size matching is important in carrying out any activity. Drug delivery is aimed at influencing the biochemistry of the body.[4]

Properties of Nanoparticulate Drug Delivery System

a. Increase of dissolution velocity by surface area enlargement: The size reduction leads to an increased surface area and thus according to the Noyes-Whitney equation to an increased dissolution velocity. Therefore Nanonization is a suitable way to successfully enhance the bioavailability of drugs where the dissolution velocity is the rate limiting step. By moving from Nanonization further down to nanonization, the particle surface is further increased and thus the dissolution velocity increases too (Fig. 1.1).
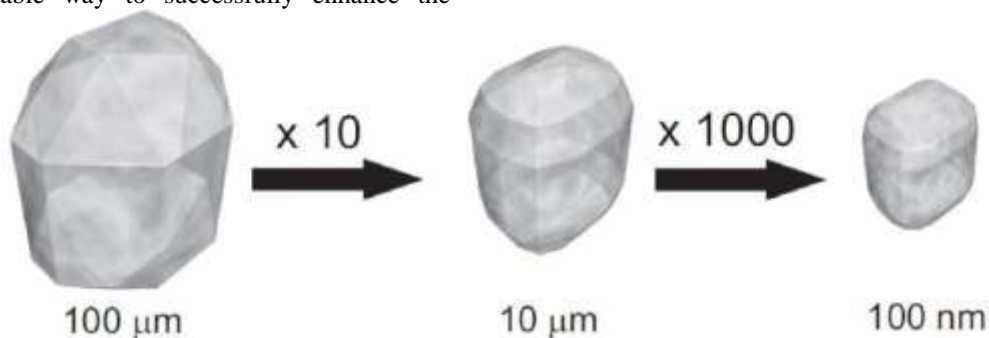

Fig. 1: Surface enlargement and increase in number of crystals by particle size diminution

b. Increase in saturation solubility

In general the saturation solubility is a constant depending on the compound, the dissolution medium and the temperature. This is valid for powders of daily life with a size in the Nanometer range or above. However, below a critical size of 1-2 μm, the saturation solubility is also a function of the particle size. The particle size reduction offers increased saturation solubility that may results in increased concentration gradient between the gut lumen and blood, consequently the absorption by passive diffusion, as seen in Fig. 1.2.[1]

As per Kelvin equation, the vapor pressure of lipid droplets in a gas phase (aerosol) increases with increasing curvature of the surface, which means decreasing particle size. Every liquid has its compound specific vapor pressure, thus the increase in vapor pressure will be influenced by the available compound specific vapor pressure. The situation of transfer of molecules from a liquid phase (droplet) to a gas phase is principal identical to the transfer of molecules from a solid phase (nanocrystal) to a liquid phase (dispersion medium), as observed in Fig. 1.3.[5]
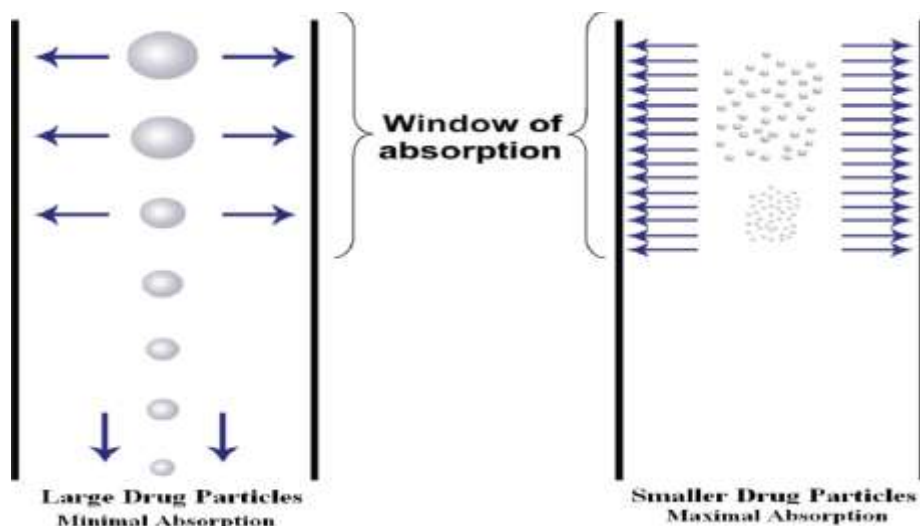

Fig. 2: Smaller Particles possess larger surface area and maximal absorption

Applications of Particulate Drug Delivery System

In last 30 years, the explosive growth of the nano- and Nano-particulate drug delivery system has burst into challenging innovations in pharmacology, which is in the process of revolutionizing delivery of biologically active compounds.[6] The main input of today"s particulate drug delivery technology is in the treatment of complex diseases.

METHODS & MATERIALS
Method
PREFORMULATION STUDY
Authentication of drug
ConfIrmation / identification of drug were carried out by following method.

UV spectroscopy[7]
Calibration curve of RLX HCl in 1% w/w Polysorbate 80

Preparation of RLF standard stock solution (100 μg/ml) in 1% w/w Polysorbate 80 A

standard stock solution of RLF HCl was prepared by dissolving accurately weighed 10 mg of RLF HCl in small amount of methanol in a 100 ml volumetric flask and the volume was made up to 100 ml by using 1% w/w Polysorbate 80 to obtain a stock solution of 100 μg/ml.

From this stock solution, aliquots with suitable dilutions were made in order to get solutions with concentrations of 2μg/ml, 4μg/ml, 6μg/ml, 8μg/ml, 10μg/ml, 12 μg/ml. The absorbance was measured at 297 nm using UV visible spectrophotometer. The standard curve was obtained by plotting absorbance v/s concentration in μg /ml.

Standard calibration curve of RLF

A standard curve of absorbance Vs concentration was plotted and found to be linear over the range of 2 to 12 μg/mL indicating its compliance with Beer's law. Results are reported in Figure 6.1
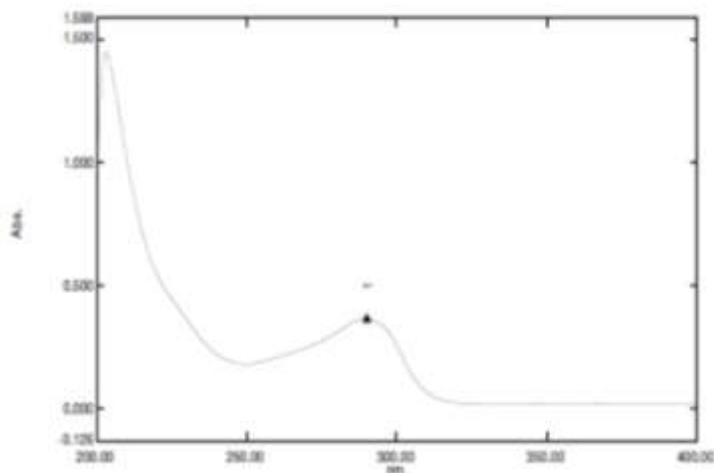

Figure 3: Calibration Curve of RLF

IR spectrum[8]

A FTIR spectrum of RLF was obtained using diffuse reflectance scan sampling method. The IR spectrum of RLF is shown in Figure 6.2.

The observed spectrum and peaks of RLF (3307, 2935, 1919, 1686, 1636, 1588 and 1217 cm^{-1}) were found.
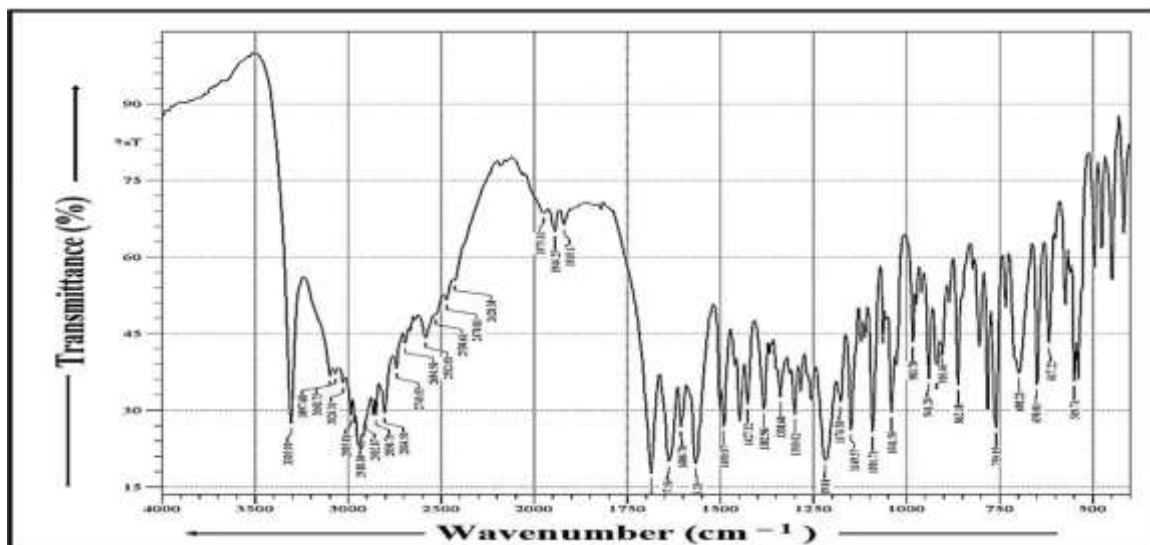
Figure 4: IR Spectra of RLF

Melting Point[9]

Melting point of RFL was determined by capillary tube method. RLF was filled into a glass capillary tube, which was sealed at one end and tied to the thermometer, placed in thiels tube containing paraffin oil. The melting point of RLF measured in laboratory was found to be in the range of 250° C to 253° C.

Solubility Study of RLF[1O]

The solubility of a substance becomes especially important in the pharmaceutical field because it often represents a major factor that controls the bioavailability of a drug substance. More over, a comprehensive knowledge of solubility permits information essential to the development and processing of its dosage forms.

Oral ingestion is the most convenient and commonly employed route of drug delivery due to its ease of administration, high patient compliance, cost effectiveness, least sterility constraints and flexibility in the design of dosage form. As a result, many of the generic drug companies are inclined more to produce bioequivalent oral drug products. However, the major challenge with the design of oral dosage forms lies with their poor bioavailability. The oral bioavailability depends on several factors including aqueous solubility, drug permeability, dissolution rate, first-pass metabolism, presystemic metabolism and susceptibility to efflux mechanisms. The most frequent causes of low oral bioavailability for a BCS class II drug is attributed to poor solubility and slow dissolution rate.

Saturated Solubility studies were performed in order to analyse solubility enhancing properties of polymers.

An excess amount of drug was added in each vials and closed with stopper. These glass vials were attached in an orbital shaking water bath. The shaking was carried out for 48 hours with the speed of 50 rpm and in the entire study the temperature was maintained around 37 ± 0.5 °C.
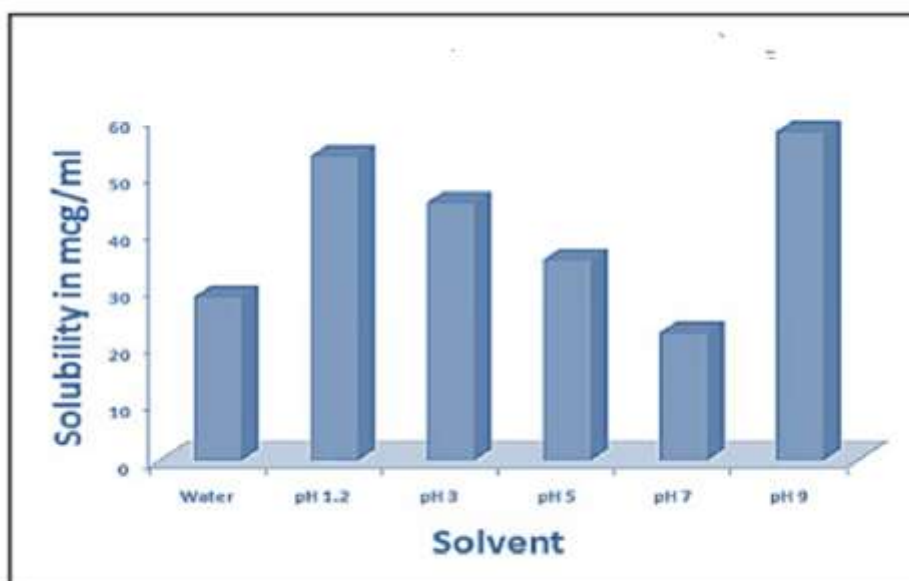The solubility of RLF is reported in Figure 6.5 and Table 6.3.

Figure 5: Solubility Study of RLF in Different Solvents

Table 1: Solubility Study of RLF in Different Solvents

Sr. No.	Solvent	Solubility (Mean ± SD)(mg/mL)
1.	Water	0.037 ± 0.0020
2.	pH 1.2 buffer	0.0435 ± 0.0011
3.	pH 3.0 buffer	0.0553 ± 0.0009
4.	pH 5.0 buffer	0.0452 ± 0.0010
5.	pH 7.0 buffer	0.0424 ± 0.0031
6.	pH 9.0 buffer	0.6182 ± 0.0262

Differential scanning calorimetry[11]

DSC thermogram of RLF is shown in Figure 6.4 The thermogram of RLF was typical of a crystalline anhydrous substance, showing onset temperature of 133° C and end set temperature of 138° C with a sharp endothermic peak at 136° C corresponding to its melting point. DSC thermogram of drug was found.
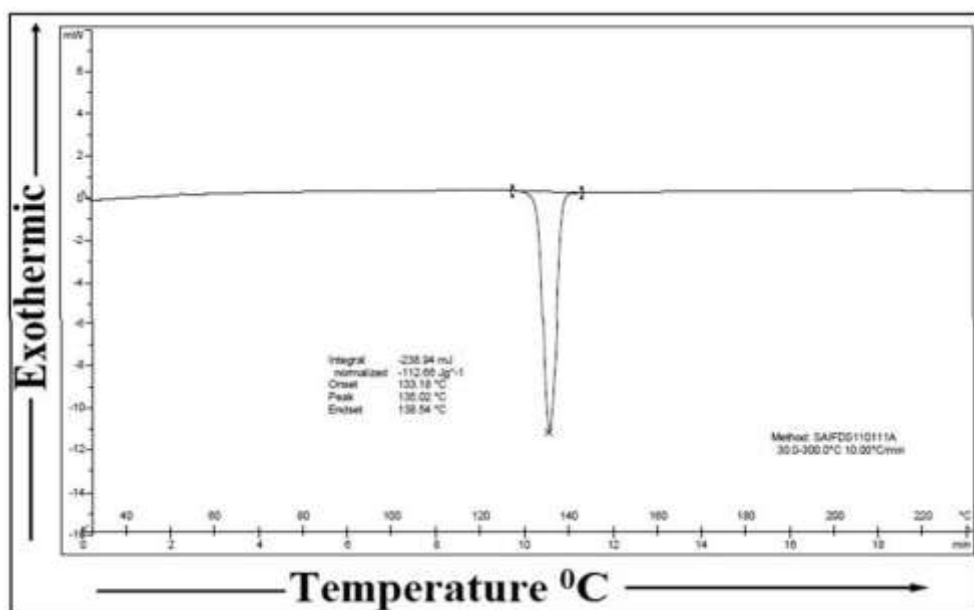
Figure 6: DSC Spectra of RLF

Scanning Electron Microscopy (SEM)

A SEM is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. Scanning electron micrographs of RLF, polymers and nanoparticles were obtained using scanning electron microscope operating at 10-kV accelerating voltage.
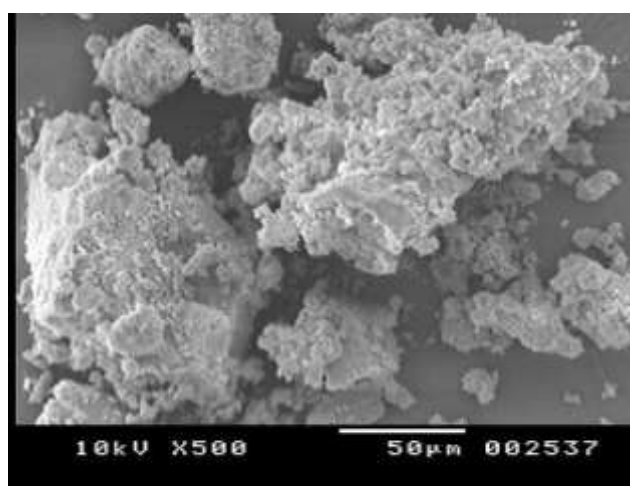

Fig. 7: SEM photomicrograph of RLF

Preparation of Nanosuspension

Nanosuspension of RLF was prepared by co-precipitation method A weighed quantity (1 % w/v) of RLF was dissolved in 100 ml of 0.1 M aqueous solution of HCl (pH 1.65) with (30% w/w) ……and/ or without stabilizers (Table 6.4). It was stirred by a mechanical stirrer (Remi Instruments, Mumbai) at room temperature. To this solution, 0.2 M aqueous solution of NaOH was added until first sign of precipitation occurs. Stirring was continued for further 15 min to get the suspension. The suspension was centrifuged at 10,000 rpm for 20

min (Refrigerated Centrifuge, Eltek, Model: RC4100D). The crystals were collected, suspended in 40 ml of distilled water and lyophilized (Khera Instruments, Mumbai) at -42°C for 72 hrs.

Table 2 : Stabilizers and their concentrations used in the preparation of nanosuspensions

Stabilizer	Stabilizer concentration (%w/w)	Drug Concentration (%w/v)
Plain Solvent (Without Stabilizer)	30	1
Polyethylene Glycol (PEG)	30	1

Kneading method (kneading mixtures, KM)[12]

The weighed quantities of the drug and PEG were taken in a mortar and triturated with a small volume (≈5 ml) of acetone. After proper trituration to smooth yellow moist mass, the mass was kneaded for 30 min and dried at 40° C till the constant weight is reached. The dried mass was pulverized and sifted through 100 and the collected powder fraction was stored in 30 ml glass vials.

Freez Drying (Lyophilation)[13]

Lyophilization of nanoparticle (NP) suspensions is a promising technology to improve stability, especially during long-term storage, and offers new routes of administration in solid state. Although considered as a gentle drying process, freeze-drying is also known to cause several stresses leading to physical instability, e.g. aggregation, fusion, or content leakage. NPs are heterogeneous regarding their physico-chemical properties which renders them different in their sensitivity to lyophilization stress and upon storage. But still basic concepts can be deducted. the most commonly investigated NP types including lipophilic, polymeric, or vesicular NPs regarding their particle properties, stabilization mechanisms in the liquid state, and important freeze-drying process, formulation and storage strategies.
The glass vials were placed on the shelves in the freeze dryer followed by slow freezing at a shelf temperature of -42°C. The samples were lyophilized for 72 h from -42° C.

In Vitro Release Studies. [14]

In vitro release studies were performed using pH 6.8 phosphate buffer containing 0.5% v/v polysorbate 80 by dialysis bag method using dialysis membrane having molecular weight of 12,000–14,000 daltons]. Accurately weighing 0.500 mg RLF Nanoparticle was filled into a dialysis membrane bag and tied at both the ends and placed in a beaker containing 100 mL of diffusion medium; temperature and speed were maintained at 37 ± 2∘ C and 100 rpm, respectively, using magnetic stirrer. Samples were withdrawn at predetermined time intervals, and the same volume was replaced with fresh buffer to maintain the sink condition. Samples were analyzed at 285 nm UV spectrophotometrically. Cumulative percentage release was then calculated from the amount of drug release.

II. RESULT & DISCUSSION
CHARACTERIZATION OF NANOPARTICLES
UV-Visible Spectroscopic method of Reloxifen

Preparation of RLF standard stock solution (100 μg/ml) in 1% w/w Polysorbate 80 A standard stock solution of RLF HCl was prepared by dissolving accurately weighed 10 mg of RLF HCl in small amount of methanol in a 100 ml volumetric flask and the volume was made up to 100 ml by using 1% w/w Polysorbate 80 to obtain a stock solution of 100 μg/ml.

From this stock solution, aliquots with suitable dilutions were made in order to get solutions with concentrations of 2μg/ml, 4μg/ml, 6μg/ml, 8μg/ml, 10μg/ml, 12 μg/ml. The absorbance was measured at 297 nm using UV visible spectrophotometer. The standard curve was obtained by plotting absorbance v/s concentration in μg /ml.
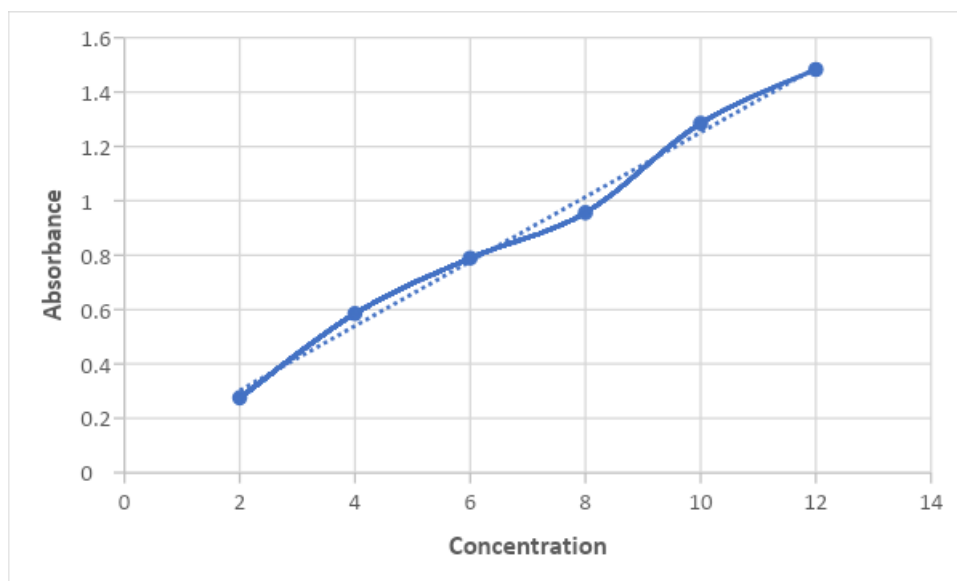
Fig. 8: Calibration curve of Reloxifen

Sr. No.	Concentration (µg/ mL)	Absorbance
1	2	0.275
2	4	0.585
3	6	0.789
4	8	0.956
5	10	1.285
6	12	1.483

Table 3 calibration curve of RFL

Infrared spectroscopic study

FTIR spectra of RLF, PEG 6000, physical mixture and nanoparticles are presented in Figure7.2. Pure RLF spectra showed sharp characteristic peaks at 3307, 2935, 1686, 1636, 1588 and 1217 cm^{-1}. All the above characteristic peaks appear in the spectra of all binary systems at the same wave number. The spectra of physical mixture and Nanoparticles were identical and the main absorption bands of RLF appeared in all the spectra in the region of C=O carboxyl group absorption around 1686 cm^{-1}. Similarly, the NH group located at 3307 cm^{-1} was not shifted. This indicated that there was no distinction between the internal structures and conformation of these samples at the molecular level.

Figure 9: FTIR spectra of (a) RLF, (b) PEG 6000, (c) KMPEG, (d) NSPEG and (d) FDPEG
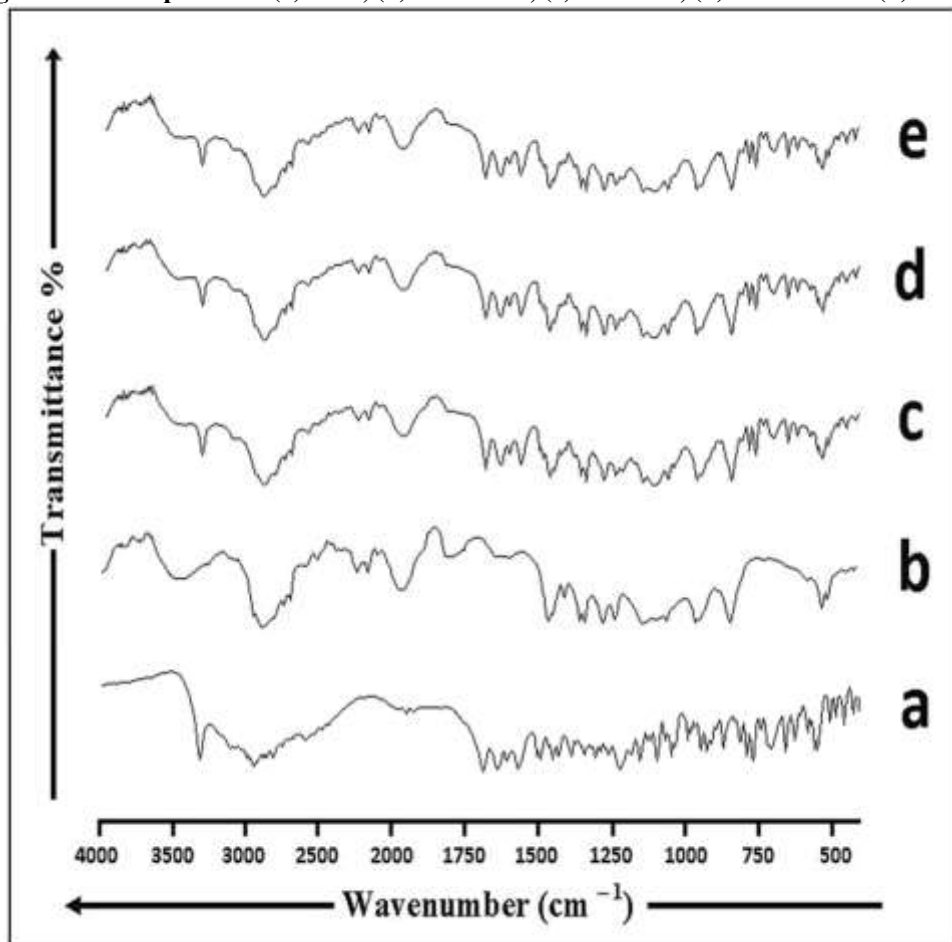

Table 4 : Interperation Of IR spectrum

Sr.No	Standard Peak (cm)	Observed Peak (cm)	Interpreation
1	1700-1500	1686	C=O
2	3300-3000	3307	N-H

Melting Point: The determined Melting point of RLF was observed in the range of

Table No 5 : Melting point

Sr.No	Drug	Melting Point	
		Reported	Observed
1	RLF	250 °C – 253 °C	250°C - 252°C

Solubility Study

Results of solubility studies are depicted in Table 7.4 and 7.5. Solubility study reveals that PEG have significant solubility enhancement property. The probable reasons for solubility enhancement by PEG can be attributed to reduction in viscosity, while swelling and water retention capacity remains unaffected. Swelling and water retention nature markedly enhances the solubility of drug due to increased surface of the carrier. This improved surface with water retention capacity helps in wetting of the hydrophobic RLF crystals and thus improving its solubility.

The enhancement of solubility by PEG can be attributed to a number of factors namely, decrease in crystallinity of drug, wetting,

solubilizing and surface active properties of polymer.

Ratio optimization[15]

Ratio optimization results are shown in Table 7.4.Although significant solubility enhancement was observed in the case of the 1: 5 ratio of RLF to PEG 6000 was considered for further study as there were insignificant differences in the solubility enhancement when the 1: 5 ratio.

This also decreased the quantity of polymer to be used

The results of the ratio optimization study gave the basis for selection of optimized drug: polymer ratio for preparation of nanoparticles with different methods like Kneading Mixture, Freez ,,,,,,,. The nanoparticles were prepared in 1:5 (RLF: Polymer) ratio with PEG 6000. Nanoparticles were prepared with these optimized ratios.

Talble 6: Ratio of Optimization

Sr. No.	Polymer	Drug to Polymer Ratio	Solubility (mg/mL)
1.	PEG 6000	1:1	0.0508 ± 0.013
2.	PEG 6000	1:3	0.0643 ± 0.008
3.	**PEG 6000**	**1:5**	**0.0889 ± 0.019**

Differential scanning calorimetry

The DSC thermograms of RLF, PEG 6000, physical mixture and nanoparticles are shown in Figure7.3. The thermal curve of RLF was typical of a crystalline anhydrous substance with a sharp endothermic peak at 136° C corresponding to its melting point, PEG 6000 exhibited characteristic peaks at 62.73^{o} C. The DSC thermogram of the KMPEG as well as NSPEG showed endothermic peaks corresponding to the melting point of RLF and PEG 6000 at 136° C and 62.73^{o} C respectively. The absence of RLF peak in case of Nanoparticles

prepared by freeze drying method could be attributed to molecular dispersion of drug in PEG 6000 and conversion of crystalline form of RLF to amorphous form which could be further confirmed by SEM studies (The thermograms showed no evidence of the formation of solid complex or any chemical interaction between drug and carrier. Collectively, these results indicated that the crystallinity of RLF was markedly affected by the method of treatment only in the presence of PEG 6000.
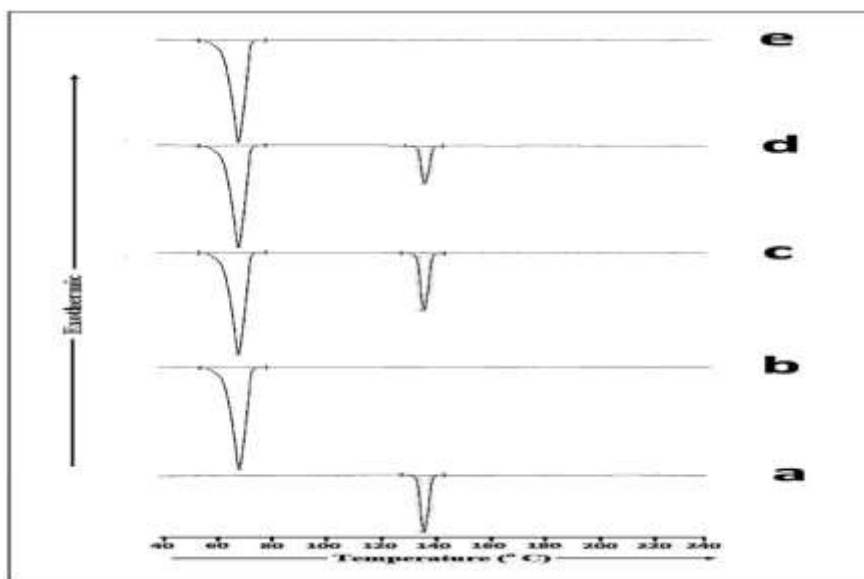

Figure 10 : DSC Thermograms of (a) RLF, (b) PEG 6000, (c) KMPEG, (d) NSPEG (e) FDPEG

Table 7:DSC Observation

Sample	Standard Temp	Observed Temp
RLF	250-253 °C	136°C
PEG 6000	58-63°C	62.73°C
KMPEG	-	136°C & 62.73°C
NSPEG	-	136°C & 62.73°C
FDPEG		62.73°C

Scanning Electron Microscopy

A SEM is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. Scanning electron micrographs of RLF, polymers and nanoparticles were obtained using scanning electron microscope operating at 10-kV accelerating voltage.

The SEM photomicrographs of RLF, KMPEG and FDPEG are shown in Figure7.4. SEM photomicrograph of PEG 6000 appeared as smooth surfaced irregular shaped particles. The SEM photomicrograph of freeze dried generated Nanoparticles appeared as smooth scaly surfaced homogeneously mixed mass. The surface morphology of NPPEG almost resembled to that of pure PEG 6000, indicating that RLF was adsorbed into the PEG 6000 and homogeneously dispersed into the polymer. SEM photomicrograph suggested formation of effective Nanoparticlesas a single component system with transformation of crystalline forms of RLF to amorphous form.
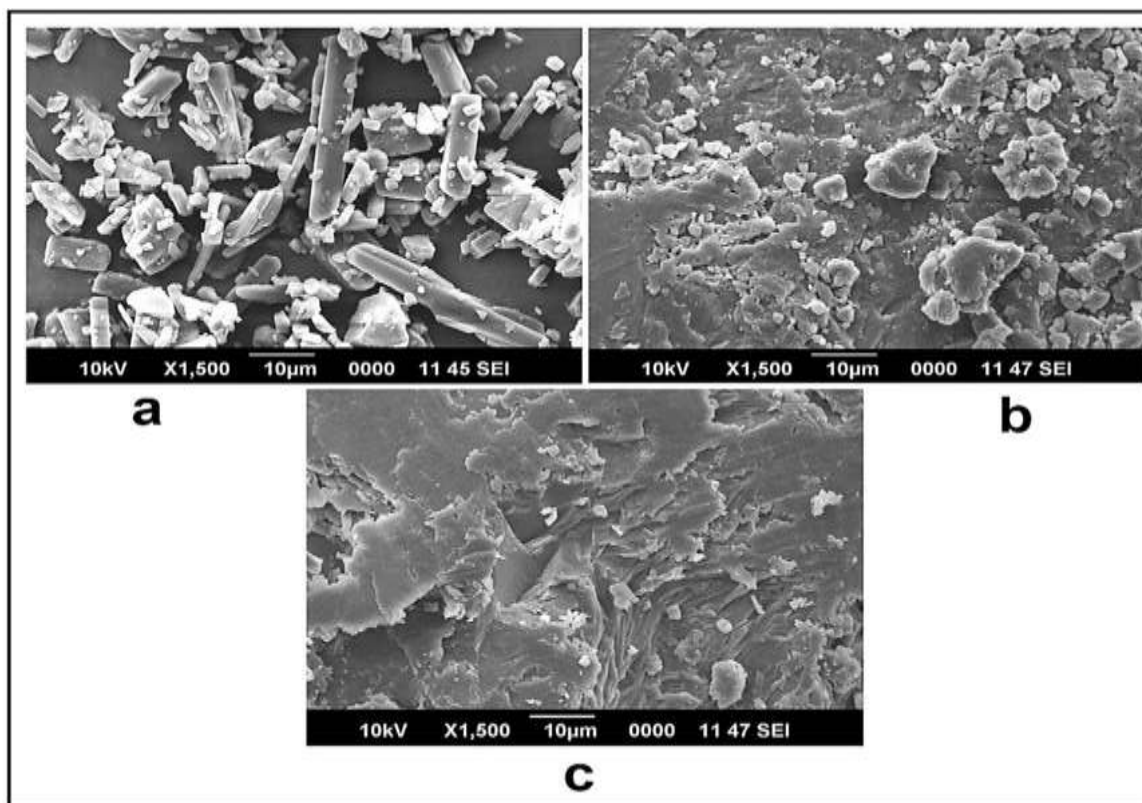

Figure 11: SEM Photomicrographs of (a) RLF, (b) KMPEG (c) FDPEG

Drug Release

Drug release rate of RLF, KMPEG and nanoparticles prepared was studied.The slight increase in the drug released rate of RLF from PEG is likely due to the ability of the polymer to enhance the wettability of the hydrophobic RLF

particles. The moderate increased drug released rate of RLF from NPPEG could be attributed to increased dispersibility and improved wettability due to presence PEG.

Table 8: Drug release Rate of RLF, KMPEG and NSPEG with FDPEG

Formulati ons	Percentage Drug Released (Mean ± S.D)								
	Time (min)								
	5	15	30	45	60	75	90	105	120
Plain RLF	5.23 ± 0.78	11.11 ± 1.45	18.09 ± 0.92	24.48 ± 1.10	29.24 ± 0.67	33.26 ± 2.26	36.09 ± 3.55	38.32 ± 2.44	39.37 ± 0.25
KMPEG (1 : 5)	7.53 ± 0.24	14.22 ± 0.87	20.91 ± 1.12	27.01 ± 2.27	32.95 ± 0.87	37.86 ± 1.34	40.99 ± 0.66	44.11 ± 0.16	45.76 ± 2.03
NPPEG	9.75 ± 0.78	15.57 ± 1.02	24.02 ± 2.34	30.72 ± 2.17	35.63 ± 1.79	40.09 ± 0.44	42.78 ± 0.69	44.57 ± 1.03	46.35 ± 0.15
FDPEG	27.19 ± 1.33	42.26 ± 0.78	52.10 ± 0.44	60.73 ± 2.20	64.76 ± 1.72	68.19 ± 2.31	70.42 ± 2.11	71.62 ± 1.33	72.36 ± 0.25

An increased drug released rate was observed from nanoparticles generated by other method, although the improvement was considerably lower than freeze dried generated nanoparticles. The significant improvement in drug released profile of freeze dried generated nanoparticles is a result of complete drug amorphization by Freeze dried improved surfactant, wetting characteristics and significant molecular dispersion of drug into carrier.

III. CONCLUSION

The solubility enhancement was observed with the 1:5 ratio of RLF to PEG 6000 was consider for further study. The absorbance was measured at 297 nm using U.V visible Spectrophotometer. The standard curve was obtained by ploting absorbance. The main absorbtion bonds of RLF appeard in all the spectra in the region of c=o carboxyl group absorption around 1686 cm^{-1} similarly, the NH group located at 3307 cm^{-1} was not shift. The melting point of RLF was observed at 252°C. The Solubility study reveals that PEG have significant solubility enhancement property. The thermal curve of RLF was observed at 136°C, PEG 6000 peak at 62.73°C, KMPEG as well as NSPEG showed similar peak to RLF & PEG 6000. The surface morphology of NPPEG almost as resembled to that of pure PEG, indicating that RLF was absorbed in PEG 6000 homogeneosly dispersed into the polymer. The significant improvement in drug released profile of freezedried generated nanoparticles is a result of complete drug amorphization by Freeze dried

improved surfactant, wetting characteristics and significant molecular dispersion of drug into carrier.

Out of all these particulate techniques, the NPs prepared by Freezdrying method exhibited fastest drug release rate and highest solubility compared to all other particulate systems prepared in the present study.

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