4. Materials and Methods

4.1 Materials

Rifabutin was gotten as a gift sample from M/s Simpex Pharma Pvt. Ltd. Kotdwar, Uttarakhand, India. Capmul MCM C-8 and Capmul MCM EP are gotten as a gift sample from Abitec Pvt Ltd. Stearic corrosive and citrus extract were acquired from SRL India. Poloxamer 188, pluronic F68, soya lecithin, sodium carboxymethyl cellulose (NaCMC) and dialysis film (atomic weight cutoff between 12000-14000) were bought from Hi-Media, India. Precirol ATO-5 got from Gattifose Pvt. Ltd. as a gift sample. Rhein, chondroitin sulfate, monosodium iodoacetate and trypsin were bought from Sigma-aldrich, India. Nanosep® was bought from Pall Life Sciences, India. Millipore Direct Q® 3UV water was utilized as a part of the considerable number of studies. Rifabutin 50 mg capsule were obtained from nearby drug store.

4.1.2 Chemicals

The following materials and equipments were used in the production of Rifabutin loaded Nanostructured lipid carriers.

Table 4.1: List of Chemicals used

Material	Source
Rifabutin	Simpex Pharma Pvt. Ltd as gift sample
Precirol® ATO 5	Gattefosse, Lyon, France
Propylene Glycol Dicaprate	Gattefosse, Lyon, France
Poloxamer 188	BASF, Germany
Methanol	Qualigens chemicals
Acetone	Qualigens chemicals
Potassium di hydrogen orthophosphate	S.D. Fine chemicals
Disodium hydrogen orthophosphate	S.D. Fine chemicals
Ammonium di hydrogen orthophosphate	S.D. Fine chemicals
Acetic acid	S.D. Fine chemicals
Purified water	Direct Q^R 3UV system of Millipore

4.1.3. Instruments

Table 4.2: List of Instruments used

Instrument	Source
Delsa Nano C	Beckman Coulter, USA
Electronic Balance ATX224	Shimadzu Corporation, Japan
CyberScan pH 510	Eutech, Thermo Fisher Scientific
Dialysis Tubing Cellulose Membrane	Sigma-Aldrich, USA
IKA® RCT Basic Magnetic Stirrer	IKA, China
Whatman filter paper	Wipro GE Healthcare Pvt Ltd, India
FT-IR spectrophotometer	Shimadzu, Japan
UV-1800	Hitachi, Japan
Syringe filter	Schleicher &Schuell , Whatman, GE Healthcare
Microlitre syringe	Paul corp.
HPLC grade water system	Merck Millipore, USA
Cooling centrifuge	REMI Laboratory Instruments, Mumbai
Micropipette (Eppendorf Research Plus)	Eppendorf, Hamburg, Germany
Refrigerator	Goderg
IKA Disperser T25 Digital	IKA, China
UP200H with Sonotrode S7	Hielscher Ultrasound Technology, Germany

4.2 Methods

4.2.1 Solubility of Rifabutin in Aqueous medium

Aqueous saturation solubility was determined in Phosphate buffer pH 7.4 solution by shake flask method. The Solution of PBS was taken 50 ml of each solution was transferred in aqueous solution of 250 ml conical flask and excess of Rifabutin was added in each. All conical flasks were kept on Rotary shaker (RemiRS-24) for overnight (Priya,et al.,2013). In between, if drug was completely dissolved in any

solution, more drug was added in the same. After that each solution was filtered through Whatman no.1 filter paper and diluted with respective media. Finally, Rifabutin was estimated at the wavelength 242 nm from UV-Visible Spectrophotometer (Velaga, et al., 2017).

4.2.2. Solubility of Rifabutin in Different Lipid

Solubility of rifabutin were evaluate in different liquid lipid such as Captex 300, Captex GTO, Captex 355, Capmul MCM-EP, Capmul MCM C-8, Labrafac, Olive Oil, Olic Acid, Precirol, Plurol Olique, Soyabean Oil, Transcutol, Triacetin were taken with 50 ml of each solution was transferred in 250 ml conical flask and excess of Rifabutin was added in each flask. All conical flasks were kept on Rotary shaker (Remi RS-24) for overnight. In between, if drug was completely dissolved in any solution than more drug was added in the same. After that each solution was filtered through Whatman no.1 filter paper and diluted with respective media. Finally, Rifabutin was estimated at the wavelength 242 nm (Nirbhavane, et al., 2017).

4.3 Analytical Method Development

4.3.1 Analytical method development

The method utilizing Acetonitrile + Methanol : Water as mobile phase yielded broad peak, whereas with Acetonitrile + Methanol : Water tailing was observed with acetonitrile and methanol as diluent. Shimadzu LC-2010C HT version 3.01 system using a C-18 column with dimensions of 250 × 4.6 mm and silica particle size of 5 µm. Isocratic elution was employed with methanol, acetonitrile and water (75:25 v/v) were used at flow rate 1ml/min-1 and wavelength is 242 nm, the symmetric peak was obtained (Sharma, et al., 2012).

4.3.2 Selecting λ_{max} for Rifabutin

The Rifabutin solution was formed for concentration of 30µg/ml solvent and 2 ml of it transferred to the cuvette in the UV-1800 Hitachi. A scan for wavelength was done from 200 to 400 nm of which peaks were observed at 2 wavelengths of 242 nm and 278 nm better peaks was observed at 242 nm and was hence chosen (Muñiz-Valencia, et al., 2008).

4.3.3 Calibration standards

Six serial dilutions were prepared in a concentration range from 10 to 100 µg/ ml. A volume of 20µl from each concentration of the solution was injected, and

chromatograms were recorded; three independent determinations were performed at each concentration. A linear calibration graph (y = 0.01x+1; where y and x are peak area and concentration, respectively) was obtained over six concentrations 10, 20, 40, 60, 80, 100 µg/ml (Trivedi, et al., 2017).

4.3.4 Analytical method validation

4.3.4.1 Accuracy

To ensure the accuracy of the analytical method, the recovery studies were carried out. The known amount of rifabutin was added to a pre-quantified sample solution of its dosage form, and the amounts of rifabutin were estimated by measuring the peak area ratios and by fitting these values to the straight line equation of calibration curve. The recovery studies were carried out three times over the specified concentration range of rifabutin. The Accuracy was calculated at three different concentrations of drug which is equivalent to 80, 100 and 120% of the active ingredient by calculating the recovery of rifabutin with %RSD (Savale, et al.,2017).

4.3.4.2 Precision:

The intra-day precision of the method was determined by repeat analysis (three identical injections) at three concentration levels. Inter-day precision was established by performing the analysis next day on a freshly prepared solution. The low RSD values indicate the ruggedness of the method (Savale, et al., 2017).

4.3.4.3 Repeatability

The peak area of 40 µgm/ml drug solution was analysed six times on the same day. The % RSD was calculated for the resultant peak area (Kumar, et al., 2011).

4.3.4.4 Robustness:

The robustness was assessed by altering the following experimental conditions such as, by changing the flow rate from 0.5 to 1.5 ml/min, the mobile phase composition with Acetonitrile + Methanol:Water (76:24, 74:26) and analysed in triplicate. In all Chromatographic varied conditions, there was no significant change in chromatographic parameters (Parikh, et al.,2011).

4.4 Selection of Ingredients of NLC

Lipids and surfactants are two main ingredients for the preparation of NLC.

4.4.1 Selection of liquid lipid

Choice of liquid lipid NLCs comprise of both solid and in addition of liquid lipids. Liquid lipids or oil for the preparation of NLCs can be selected in light of the relative drug solubility. The method embraced for the determination of solubility of drug in oil was similar to the method utilized as a part of microemulsions or self emulsifying system. The fixed volume (1 mL) of various oils was gone up against 2 mL glass vials independently. An excess amount of drug was added to every vial and kept in shaker incubator for 72hr at 37◦C and 150 RPM. The vials containing oil and drug were centrifuged at 5000 RPM for 30 min to isolate the undissolved drug and solubility of drug was determined in oils using HPLC (Babazadeh, et al.,2017).

4.4.2 Selection of Solid lipid

Choice of solid lipid for the preparation of NLCs, solid lipid can be shortlisted according to their relative affinity for the drug. Various methods have been utilized already for the determination of solubility of drug in solid lipids. In any case, we have developed another method in view of the standard of drug portioning amongst water and solid lipid (at the temperature above melting point of the lipid) according to their affinity of different phase. Moreover, solid lipids were assessed for their physical compatibility with the liquid lipid (Katouzian, et al., 2017).

4.4.3 Affinity Study

To 10 mL of water in test-tube, excess amount of rifabutin measure and added to accomplish the saturation solubility. Excess drug was removed by filtration and 100 mg of solid lipid was added to each test-tube. All the test-tubes were kept in shaking water shower at 75^0C for 1 h took after by cooling at room temperature. The solidified solid lipids were removed and dissolved in Methylene chloride independently. Drug was determined in solid lipid solution and in water as well. Drug was determined by HPLC to calculate the percentage of the drug entrapped in solid lipid (Lasoń, et al., 2017).

4.4.4 Physical compatibility of solid and liquid lipid

Solid lipids with with maximum affinity with the drug were blended with the liquid lipid in a proportion 1:1 in various glass tubes. The lipid mixture was dissolved, shaken and permitted to solidify at room temperature. The glass tubes were analyzed visually for the nonappearance of separate layers in solidified lipid mass. Moreover, the smeared of

solid-liquid lipid were spread over a glass slide and inspected microscopically (Wissing, et al., 2004).

4.4.5 Selection of solid–liquid lipid ratio

The proportion between the weights of solid and liquid lipids was chosen based on the bases of melting point of mixture. Selection of solid and liquid lipids were mixed in the proportion of ranging from 80:20 to 20:80, melted for 01 hr and congealed at room temperature. The melting point of the congealed lipids was determined by the capillary method. The melting point of selected ratio was further conformed by DSC. The solid sample of (05mg) of binary mixture of solid and liquid lipid and in addition unadulterated solid lipid were examined on a DSC Differential Scanning Calorimeter at a scanning rate of 5^0C/min over the temperature range of 30-100^0C (Hentschel, et al.,2008).

4.4.6 Selection of surfactant

Identification of Surfactant for the Preparation of NLCs was chosen on the bases of its ability to emulsify solid-liquid binary lipid (SLB). 100 mg of SLB was disintegrated in 3 mL of methylene chloride and added to 10 mL of 5% surfactant solution under magnetic stirrer. Organic phase was removed at 40^0C and the resultant suspensions were diluted with 10-fold Milli-Q water. Percentage transmittance of the resultant sample was observed utilizing UV spectrophotometer at 242 nm (Kruk, et al.,2012).

4.5 Preparation of Nanostructure Lipid Carriers of Rifabutin

NLC's were chosen to be prepared by Solvent Injection technique developed by (Muller et al, 2002) based on literature review. Although several processes and formulation parameters were changed batch to batch, but the general procedure was outlined here. 5mg of Rifabutin was weighed and dissolved using bath sonicator in liquid lipid (µl) taken in a 25 ml beaker (Škalko-Basnet, et al., 2017) weighed amount of solid lipid (mg) was added and also measured 10ml of acetone (ml). Total lipid concentration was maintained constant at 1%w/w. The beaker was covered tightly with an aluminium foil and sonicated in a preheated bath sonicator or about 2 min. Parallel to this 20 ml of poloxamer 188 solutions (1.5%w/w) was pipetted into a 50 ml beaker and heated to 80ºC on a hot plate placed under a IKA T25 disperser (off mode) (Harisa, et al.,2017). The hot acetone solution (~38ºC) was withdrawn into a 6 ml syringe and disperser turned on.

The acetone solution was added dropwise to the poloxamer solution stirred constantly at 10,000/15,000 rpm. The procedure was executed as directed by design of experiment. The formed pre-emulsion was cooled for about 2 minutes prior to sonication using UP200H sonicator. The formed nanoformulation was filtered prior to storage (Gaspar, et al., 2017).

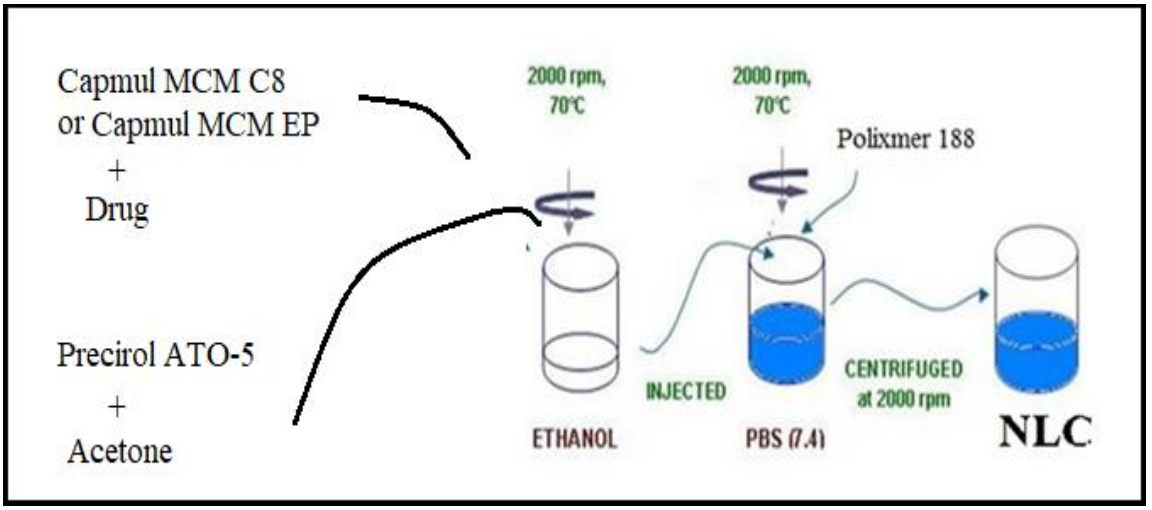

Fig:4.1- Diagrammatic presentation of NLC Method for formulation Preparation

4.5.1 Process of Screening through Plackett-Burman Design (PB)

In the process of Screening and Optimisation can evaluate how the regression tool in Mintab-17, intended for response surface methods (RSM), for this work with such happenstance variables if there's any possibility of performing a designed experiment. Highly Fractionated experiment makes good building blocks for sequential experiments. Many researches use Plackett-Burman design for this purpose by Minitab-17 software. In this work, I confounded in the interpretation of effects from these low-resolution designs. The main effect may be aliased with plausible two factor interactions. If this occurs, we might be able to eliminate the confounding by running further experiments using a fold over design. This technique adds further fractions to the original design matrix (Gaspar, et al.,2017).

Screening was done indirectly using screening method involving 2 levels in these techniques. Of all the factors affecting the process of solvent injection namely: temperature, size of needle, beaker size, sonication time, sonication amplitude, sonication, pulse time, homogenization speed, homogenization time, injection speed, intermittent cooling time etc. Using Minitab-17, a method for screening the factors was chosen from set of available methods namely:

1) Regular 2-level Factorial design (Res III, Res IV, Res V)
2) Minimum-Run Resolution V Characterization Design
3) Resolution 5 Irregular Fraction Design
4) Minimum-Run Resolution IV Screening Design
5) Definitive Screening Design
6) Plackett-Burman Design

Of all the above listed methods Irregular Res V design *[Irregular Res V (4-11 factors) – These are a special set of Resolution V designs, usually with fewer runs or more power than a regular fractional factorial to estimate main effects and two-factor interactions*] was opted for screening using no blocks, 10 Factors with 2 responses (Particle Size and Entrapment Efficiency) and 3 replicates rendering 12 runs in all with main effects aliased with 3 Factor interactions (FI's). 3 FI's were assumed to be negligible although 2 FI's were not done away with and were fully ascertained using this design (Kumar, et al.,2017). Selected factors are evolved in this study on the basis of Ishikawa diagram and shown in table given below:

Table: 4.3- Randomized Design Table of selected factors from Minitab-17

S. No	Temperature	Needle Size	Injection Speed	Homogenization time	Homogenization Speed	Sonication time	Drug Lipid Ratio	Solid Lipid & Liquid Lipid Ratio	Poloxamer 188	Organic & Aqueous Ratio
1	-	-	-	-	-	-	-	-	-	-
2	+	-	+	-	-	-	+	+	+	-
3	+	-	-	-	+	+	+	-	+	+
4	+	-	+	+	-	+	-	-	-	+
5	-	+	+	+	-	+	+	-	+	-
6	-	-	-	+	+	+	-	+	+	-
7	-	+	-	-	-	+	+	+	-	+
8	+	+	+	-	+	+	-	+	-	-
9	+	+	-	+	+	-	+	-	-	-
10	-	-	+	+	+	-	+	+	-	+
11	-	+	+	-	+	-	-	-	+	+
12	+	+	-	+	-	-	-	+	+	+

4.5.2 Process of Optimisation through Box-Behnken Design

Another class of response surface designs is called Box-Behnken designs. They are very useful in the same setting as the central composite designs. Their primary advantage is in addressing the issue of where the experimental boundaries should be, and in particular to avoid treatment combinations that are extreme. By extreme, we are thinking of the corner points and the star points, which are extreme points in terms of region in which we are doing our experiment. The Box-Behnken design avoids all the corner points, and the star points (Muthukumar, et al., 2003). One way to think about this is that in the central composite design we have a ball where all of the corner points lie on the surface of the ball. In the Box-Behnken design the ball is now located inside the box defined by a 'wire frame' that is composed of the edges of the box. If you blew up a balloon inside this wire frame box so that it just barely extends beyond the sides of the box, it might look like this, in three dimensions. Notice where the balloon first touches the wire frame; this is where the points are selected to create the design (Sun, et al.,2010).

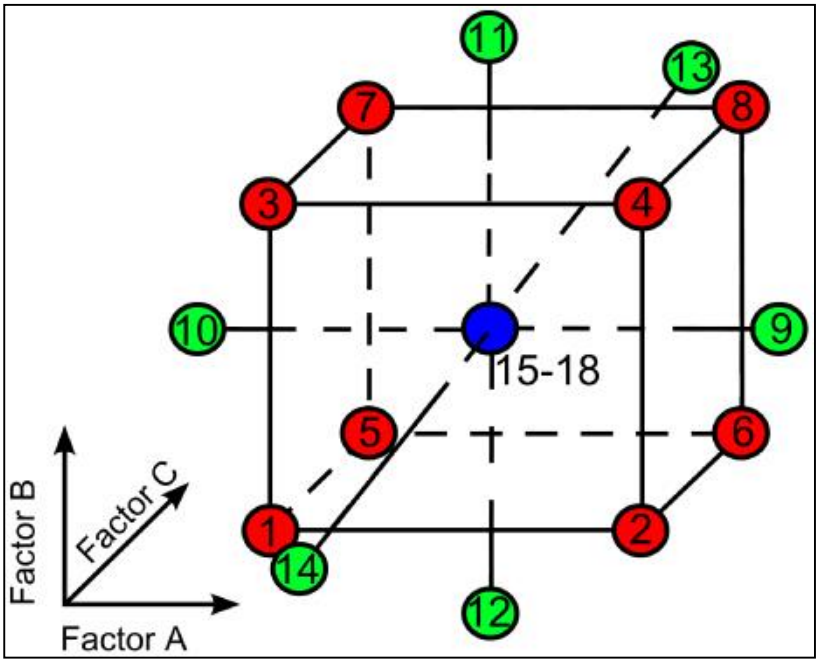

Fig:4.2- Box-Behnken design for three factors shows the geometric representation

Therefore the points are still on the surface of a ball, but the points are never further out than the low and high in any direction. In addition, there would be multiple center points as before. In this type of design you do not need as many center points because points on the outside are closer to the middle. The number of center points are again chosen so that the variance of is about the same in the middle of the design as it is on the outside of the design. In Minitab we can see the different designs that are available. Listed at the

bottom are the Box-Behnken Designs (Phatak, et al.,2013).

Fig:4.4-Box-Behnken design for three factors shows the design

S.NO	Homogenization time	Poloxamer 188	Organic: Aqueous Ratio	Solid Lipid: Liquid Lipid Ratio
1	0	-1	1	1.5
2	0	1	1	-1
3	-1	-1	0	1.5
4	1	1	10	-1
5	0	0	-1	-1
6	0	0	10	1
7	-1	-1	0	1
8	0	0	0	1.5
9	0	1	0	1.5
10	0	-1	-1	1.5
11	1	-1	0	1
12	1	0	0	0
13	1	-1	0	1.5
14	-1	0	0	-1
15	0	0	1	-1
16	-1	-1	1	-1
17	0	1	0	1
18	0	-1	1	1
19	1	-1	-1	-1
20	1	-1	1	-1
21	0	1	-1	-1
22	0	-1	-1	1
23	-1	-1	-1	-1
24	0	-1	0	-1
25	0	-1	0	-1
26	-1	1	0	-1
27	0	-1	0	-1

A Box-Behnken (BB) design with two factors does not exist. With three factors the BB design by default will have three center points and is given in the Minitab output shown above. The last three observations are the center points. The other points, you will notice, all include one 0 for one of the factors and then a plus or minus combination for the other two factors. If you consider the BB design with four factors, you get the same pattern where we have two of the factors at + or -1 and the other two factors are 0. Again, this design has three center points, and a total of 27 observations (Aslam, et al.,2016).

Comparing the central composite design with 4 factors, which has 31 observations, a Box-Behnken design only includes 27 observations. The CCD and the BB design can work, but they have different structures, so if your experimental region is such that extreme points are a problem then there are some advantages to the Box-Behnken. Otherwise, they both work well. The central composite design is one that I favor because even though you are interested in the middle of a region, if you put all your points in the middle you do not have as much leverage about where the model fits. So when you can move your points out you get better information about the function within your region of experimentation (Garg, et al.,2017). However, by moving your points too far out, you get into boundaries or could get into extreme conditions, and then enter the practical issues which might outweigh the statistical issues. The central composite design is used more often but the Box-Behnken is a good design in the sense that you can fit the quadratic model. It would be interesting to look at the variance of the predicted values for both of these designs. (This would be an interesting research question for somebody) The question would be which of the two designs gives you smaller average variance over the region of experimentation (Vasantharaja, et al.,2017).

The usual justification for going to the Box-Behnken is to avoid the situation where the corner points in the central composite design are very extreme, i.e. they are at the highest level of several factors. So, because they are very extreme, the researchers may say these points are not very typical. In this case the Box Behnken may look a lot more desirable since there are more points in the middle of the range and they are not as extreme (Baruah, et al.,2017). The Box-Behnken might feel a little 'safer' since the points are not as extreme as all of the factors.

4.6 Characterization of Nanoformulation

4.6.1 Particle size and Zeta potential analysis of optimized batch:

Particle Size and zeta potential of nanoformulation were measured by Delsa Nano C (Beckman Coulter Counter, USA). It works on the principle of Photon Correlation Spectroscopy (PCS) for particle size and Electrophoretic light scattering for zeta potential. Conventional static and automatic titration measurements for both size and zeta potential distributions of suspended particles in a wide range of size and concentration. The Delsa Nano also can measure zeta potential of a solid surface or film (Rawat, et al.,2011).

4.6.2 Particle size determination

More precisely, this is done by measuring the angular distribution of time-dependent scattered light intensity due to density and/or concentration fluctuations. From these fluctuations, an autocorrelation function is derived, which is inverted to determine the diffusion coefficient of the analyzed sample. The diffusion coefficient in turn represents the velocity of the analyte's Brownian motion. The size of the analyte is now calculated based on the measured velocity with respect to two further factors having significant impact on this calculation; medium viscosity and temperature (Yasir, et al., 2014).

The prepared rifabutin NLC was dispersed in water by sonication and vortex mixing for 30 seconds and the particle size (Z-average mean) and zeta potential were determined by using Delsa Nano C (Beckman Coulter Counter, USA). For the size distribution studies, the NLC were diluted in sterile filtered, highly purified water and measured in concentrations between 30 and 100 µg/ml (González-Mira, et al., 2011). The experiments were performed at room temperature or set to 25^0C and scattering angle of 90^o, in Nanosizer ZS experiments. Similar procedure was performed to determine the particle size of the NLC containing rifabutin. All assessments were executed in triplicate (n = 3), and the standard deviation (±SD) was noted (Mishra, et al., 2015).

4.6.3 Zeta potential

Zeta potential was estimated utilizing a disposable zeta cuvette. The instrument is a laser-based multiple angle particle electrophoresis analyzer. Using Doppler frequency shifts in the dynamic light scattering from particles, the instruments measure the electrophoretic mobility (or zeta potential) distribution together with the hydrodynamic

size of particles (size range -20 to -30 mv) in liquid suspension by Photon correlation spectroscopy Measurement (Ferreira, et al., 2015).

All assessments were executed in triplicate (n=3), and the standard deviation (±SD) was noted for both the formulations. Similar procedure was performed to determine the particle size of the Rifabutin loaded Nano Lipid Carrier (Mishra, et al., 2015).

4.6.4 Surface Morphology characterized by Scanning Electron Microscopy (SEM)

The morphology of the NLC was studied by Scanning Electron Microscopy. Samples were prepared by allowing the sample air dried on a stuck to an aluminum stub. The stubs were than coated with gold to a thickness of 200 to 500 A^0 under an argon atmosphere using a gold sputter module in a high vacuum evaporator. The coated samples were randomly scanned and photograph was taken with SEM (LEO-430, Cambridge, U.K) from the Central Instrument Lab Facility, IIT-BHU, Varanasi (Goto, et al., 2007).

4.6.5 Surface Characterization by Transmission Electron Microscopy (TEM)

External morphology of prepared NLC's was determined by using Transmission electron microscopy (TEM). Rifabutin loaded NLC's (optimized batch) were placed on a carbon coated copper grid and then a drop of 1% phosphotungstic acid was added to stain the nanoparticles. The superfluous phosphotungstic acid on nanoparticles was wiped off by the filter paper. The TEM images were obtained by using Transmission electron microscope (TECHNAI 12 G^2 FEI Netherland, Department of Anatomy IMS-BHU) (Pugh, et al.,1983).

Encapsulation efficiency of Nano Lipid Carrier (NLC)

Encapsulation efficiency of Nano Lipid Carrier (NLC) was calculated with the help of Nanosep method. A known dilution of the NLC was prepared and 500µl of it was transferred to the upper chamber of Nanosep® centrifuge tubes fitted with an ultrafilter. The Nanosep® was centrifuged at 15,000 rpm for 20 mins. The filtrate was diluted appropriately and the amount of drug in the phase was determined using UV spectroscopy analysis at 242nm (Phatak, et al.,2013). The entrapment efficiency was calculated by the following equation:

$$\text{Drug E.E. (\%)} = \frac{\text{Total drug content-Free drug content}}{\text{Total drug content}} \times 100$$

Total drug content = conc. obtained × dilution factor × Volume of formulation

4.6.6 Determination of total drug content of Nanoformulation:

The total drug amount in nanoformulation was determined by spectrophotometrically (UV-1800 HITACHI). A 1.0 ml aliquot of nanoformulation was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in methanol and filtered with a 0.45µm filter, and Rifabutin content was assayed spectrophotometrically at 242 nm (λ_{max}) (Bondì, et al., 2014). The calculation was performed as follows:

$$\text{Total drug content} = \frac{\text{Vol. total} \times \text{Drug amount in aliquot}}{\text{Vol. aliquot}}$$

4.6.7 *In vitro* drug release study

The dialysis bag diffusion technique was used to study the in- vitro drug release of Rifabutin formulation. 01 ml of Rifabutin NLC Preparation was placed in the dialysis bag (cellulose membrane, molecular weight cut off 12,000) hermetically sealed and immersed into 50 ml of phosphate Buffer Saline (pH 7.4). The entire system was kept at 37 ± 0.5 °C with continuous magnetic stirring at 200 rpm/min. Samples were withdrawn from the receptor compartment at predetermined time intervals and replaced by fresh medium (Jaafar-Maalej, et al., 2011). The amount of drug dissolved was determined with UV spectrophotometer at 242 nm. Rifabutin has very low solubility in phosphate buffered saline (pH 7.4). Sink conditions were maintained for release studies ($C_{1<}$ C_{s1} ×0.2). Therefore the final concentration of Rifabutin after the complete in phosphate buffer saline (pH 7.4) was maintained less than 28 µg/ml, in compliance with the sink conditions (Wissing, et al., 2004).

C_1 = final concentration of Rifabutin after the complete release of the drug in the phosphate buffer saline (pH 7.4).

C_{s1} = saturation solubility of Rifabutin in the phosphate buffer saline (pH 7.4)

Since Rifabutin is insoluble in water, Rifabutin solution (10 mg/100ml) in 50% v/v mixture of methanol and water were used as controls for *in-vitro* release studies.

4.6.8 Release kinetics (Kinetic model analysis and statistics)

Zero order kinetics:

Drug dissolution from pharmaceutical dosage forms that do not disaggregate and release the drug slowly (assuming the area does not change and no equilibrium conditions are obtained) can be denoted by the following equation:

$$f_t = K_0 t$$

where f_t represent the fraction of drug dissolved in time t and K_0 apparent dissolution rate constant or zero order release constant.

First order kinetics:

The pharmaceutical dosage forms following this dissolution profile, such as water insoluble drugs from porous matrices, release the drug remaining in its interior, in such a way, that the amounts of drug released by unit of time diminish (Singh, et al., 2011).

$$log\ Q_t = log\ Q_0 + K_1 t\ /0\ 2.303$$

Where,

Q = Amount of drug released in time t,

Q_0 = Initial amount of drug in the solution

K =First order release constant.

Higuchi model:

Higuchi developed several theoretical models to study the release of water soluble and low soluble drugs incorporated in semi-solid and/or solid matrixes. Mathematical expressions were obtained for drug particles dispersed in a uniform matrix behaving as the diffusion media. To study the dissolution from a planar system having a homogeneous matrix, the relation obtained was the following:

$$f_t = Q = \sqrt{2D(C - Cs)Cs\ t}$$

Where, Q = Amount of drug released in time t per unit area,

C = Drug initial concentration,

C_s = Drug solubility in the matrix media,

D = Diffusivity of the drug molecules (diffusion constant) in the matrix substance.

Korsmeyer–Peppas model:

This model is a simple, semi-empirical model, relating exponentially the drug release to the elapsed time (Andrés, et al. 2017):

$$f_t = at^n$$

where, a is a constant incorporating structural and geometric characteristics of the drug dosage form, n is the release exponent, indicative of the drug release mechanism, and the function t is fractional release of drug (Kruk, et al., 2012; Nagaich, et al., 2016).

4.7 Cell Culture Studies

4.7.1 Establishment and maintenance of cell lines

A human macrophage cell line (J7441.A) (purchased from NCCS Pune) was grown as monolayer using RPMI supplemented with 5% fetal bovine serum. The media was supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin to depress the growth of micro-organisms. Cell lines employed in this study was maintained in a humidified incubator at 37±2^{o}C with 95% air and 5% CO_2. Cells were sub-cultured twice weekly by simply re-suspending the cells using 0.2 % Trypsin 0.025% EDTA and then replacing half of the cells suspension with a fresh medium. Adherent cells were grown to 80% confluence in tissue culture grade flasks and were sub cultured by discarding the used medium, leaving the cells adhered to the bottom of the flask (Lee, et al., 2005; Park, et al., 2003).

4.7.2 Viable cell counts

Viable and non-viable cells were distinguished using the hemocytometer, for this purpose, trypan blue was used. Live cells can exclude the trypan blue stain, leaving them with a normal appearance under the microscope. Dead cells, however take up the stain making them appear blue. An equal volume of cells and stain (0.4%) were mixed and applied to the bright-line haemocytometer. The number of cells counted (ignoring blue cells) was multiplied by $2x10^4$ to calculate cells/ml, thus taking into account the dilution factor upon addition of the trypan blue (Cappelier, et al.,1999; Fukushima, et al., 2007; Kastbjerg, et al., 2009).

4.7.3 Cell Cytotoxicity Studies

4.7.3.1 (Modified Microculture Tetrazolium Assay)

The modified microculture tetrazolium assay was used to assay the IC_{50} (drug concentration that caused 50% inhibition of cell proliferation/cytotoxicity) of Rifabutin on J 7441.A macrophage cells in both the formulations i.e. both formulations. Briefly, cells harvested from exponential phase cultures were counted by trypan blue exclusion (only cell preparations demonstrating viability >90% were used) and dispensed within 96-well flat-bottomed Costar (Cambridge, MA) culture plates (10000 cells/100 µl per well for a 3-day incubation) (Hillegass, et al., 2010;Kastbjerg, et al., 2009; Pieters, et al., 1990;Ulukaya, et al.,2004).

These cells were exposed to serial concentrations of the indicated drug (20-0.5 µg/ml of control formulation and 7.5-0.5 µg/ml of formulation in 100 µl of culture medium, RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum), over a 3-day incubation at 37°C, 5% CO_2, and 95 % relative humidity. After incubation, 50 µl of modified microculture tetrazolium {5 mg [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]/ml PBS} was added and further incubated for 4 h at 37°C (Hillegass, et al., 2010).

Medium was aspirated slowly through a blunt 18-gauge needle leaving ~20 µl of the supernatant, and the reaction product thoroughly solubilized with 150 µl of dimethyl sulfoxide. The plates were read spectrophotometrically at excitation & emission wavelength 412 & 661 nm in a Titertek multiscan multiplate reader (Flow Laboratories, Mississauga, Canada). Cytotoxicity was expressed in terms of percentage of control absorbance (mean ± S.D.) after subtraction of background absorbance. The IC_{50} was determined from a linear regression curve (plot of percent control absorbance versus log drug concentration) of the data from studies completed in triplicate (Wang, et al., 2012).

$$\text{Relative viability} = \frac{(\text{Experimental absorbance-background absorbance})}{(\text{Absorbance of untreated controls-background absorbance})} \times 100\%$$

4.7.4 Spectrofluorimetric Analysis

4.7.4.1 Cell Uptake Studies

Calcein loaded NLCs were prepared to study their uptake to Macrophage cell line J7441.A. Plain calcein loaded NLCs with both type of formulation were prepared (Capmul MCM EP

and Capmul MCM C-8) were assayed. The calcein loaded NLCs were prepared by similar method. The calcein is used in concentration 0.5mg/ml in PBS instead of drug. The calcein loaded NLCs were purified from unloaded calcein with Sephadex G-50 column (Fan, et al., 2001).

Both type of formulations were diluted in 1 ml RPMI solution and added to monolayers of J7441.A macrophage cell lines ($2x10^5$) grown in 96 wells culture plate and incubated for 30, 60, 120, 180 and 240 min at 37ºC. Cell monolayers were thoroughly washed in phosphate buffered saline (PBS), and lysed in 0.75 ml of lysis buffer (PBS containing 0.1% Triton X-100). The fluorescence was measured in the lysis buffer extract using Shimadzu fluorescence spectrophotometer at excitation and emission wavelength of 510/661nm (Horvath, et al., 2011).

The cell surface-bound NLCs were stripped by two washes with acidic saline (0.9% NaCl adjusted to pH 3 with acetic acid) followed by two washes with PBS to determine the fraction of internalized NLCs. The remaining fluorescence was considered as internalization. Data of time kinetics of fluorescence related cell uptake study of internalized formulations was recorded (Chao, et al., 2008;Csaba, et al., 2005;Hentschel, et al., 2008).

4.7.4.2 Fluorescent Microscopy

Fluorescent Microscopy was done on both NLCs formulation. Cells were cultured in 6-well plastic dishes containing 18mm cover slips for 24 hr. Calcein loaded NLCs were added to the cell culture media RPMI, at a particle concentration of 0.10 mg/ml. After 3 h of incubation at 37^0C, the cells were washed six times with PBS. The cover slips were put on slides coated with buffered mounting medium and viewed by Olympus fluorescence microscopy (Japan) as shown in Photomicrograph (Juette, et al., 2008).

4.8 *in-vivo* Study

4.8.1 Animal Care and Handling

The animal study protocol was employed during this work after obtaining the requisite approval of the study protocol by the Institutional Animal Ethics Committee with protocol no. Dean/2015/CAEC/1417. The *Wistar rats* were quarantined in the animal house maintained at 20± 2.0 and 50%-60% RI-I. A 12-h dark/light cycle was maintained throughout the study. Rats had free access to food (pellet diet supplied) and distilled water *ad libitum*. Twenty four rats weighing 250±20-

300±20g were kept for overnight fasting. Animals were divided into Four groups comprising six animals in each group (n=6). The three groups were treated orally with pure drug (dispersed in 0.3% sodium CMC), prepared NLC Capmul MCM EP and NLC Capmul MCM C-8 with the dose of 15mg/kg body weight of animals. Corresponding volumes (approximately 1 to 2 ml of NLC formulation or drug dispersion) were administered to the rats calculated on the basis of individual body weight (Arnemo, et al., 2017;Sikes, et al., 2011).

4.8.2 Pharmacokinetic study

Single dose pharmacokinetic study was performed by collecting blood samples from the retro-orbital plexus of rats. On the first day of treatment blood samples (0.25 to 0.3 ml) were collected in heparin-coated micro centrifuge tubes at pre-determined fixed time points after administration of drug are 0, 06, 12, 24, 36, 48 and 60 hrs. respectively and NLCs from the rats of respective groups. Plasma was collected from blood samples by centrifugation at 2000xg for 5 min and was stored at -40 $^{\circ}$C until analyzed (Sousa, et al., 2008;Weiner, et al., 2005).

4.8.3 Pharmacokinetic analysis

Different pharmacokinetic parameters for drug dispersion as well as for prepared NLC formulations were calculated based on non-compartmental model using Kinetica 5.0 software. Finally, all pharmacokinetics parameters as $T_{1/2}$, Cmax, Tmax, AUC_{Last}, AUC_{Total} AUMC and MRT were compared and plasma concentration profiles were plotted (Hamzeh, et al., 2003;Moyle, et al., 2002).

4.8.4 *In-Vivo* Serum and Organ Distribution Studies

Mice were prepared by injecting Plain rifabutin by intravenously into mice. The Albino mice was taken an average weight size 250 to 300 mg for present study were divided into four groups with six mice in each group. They were fasted overnight before administration of dose. To the first group, aqueous solution of rifabutin was administered intravenously (via the tail vein). The dose given for mice was 5.0 mg/kg body weight. To the second and third group of mice, NLC of rifabutin with Capmul MCM EP and NLC containing rifabutin with Capmul MCM C-8 formulations were administered respectively, fourth group was kept as control (Liu, et al., 2010;Liu, et al., 2009).

One mice from each group was sacrificed after 2 hr, 8 hr, 20 hr, 40 hr and 60 hr and after administration of the formulations. Blood was collected by cardiac puncture.

Different organs i.e. heart, liver, spleen, kidney lung were excised, isolated and dried with tissue paper and weighed. The amount of drug present in plasma and organs was determined by HPLC method (Verma, et al., 2012).

4.8.5 Hemolysis Study

Drug-induced hemolysis is a relatively rare but serious toxicity liability. It occurs by two mechanisms:

- Toxic hemolysis:- direct toxicity of the drug, its metabolite, or an excipient in the formulation.
- Allergic Hemolysis:- toxicity caused by an immunological reaction in patients previously sensitized to a drug.

Although the majority of normal individuals may suffer toxic hemolysis at sufficiently high concentrations of hemolytic drugs, for most drugs toxic hemolysis involves lower doses given to individuals who are genetically predisposed to hemolysis (Rolling, et al., 2013).

4.8.6 *In-Vitro* Hemolysis Study

In vitro hemolysis assay was carried out as the available literature reported. In brief, rat blood samples were collected from healthy rats. Fresh EDTA-stabilized whole blood samples were used. The red blood corpuscles (RBCs) were separated by centrifuging 1 mL blood 1:3 (v/v) with PBS at 500g for 10 min. The purification process was repeated for four times. Then the washed RBCs were diluted to 10 mL in PBS. To test hemolytic activity of Plain Drug (Karpman, et al., 1998), NLC of Capnul MCM EP and NLC containing Rifabutin with Capmul MCM C-8. We exposed 1 mL of RBC suspension (±0.4×108 cells/mL) to different concentrations of samples in PBS. RBCs were suspended in deionized water and PBS to obtain positive and negative controls, respectively. Samples were incubated in rocking shaker at 37^0C for 4 h. Incubation was followed by centrifugation at 10000g for 10 min. Absorbance of hemoglobin was monitored at 540nm, with 655nm as a reference, in a microplate spectrophotometer (BioTek, model Power Wave XS2, Medispec, India) at 37 °C (Liu, et al., 2003). Percent hemolysis was determined using the following equation:

$$\% \text{ Hemolysis} = \frac{\text{Sample Abs 540-655nm - Negative control Abs 540-655nm}}{\text{Positive control Abs 540-655nm - Negative control Abs 540-655nm}} \text{ X } 100$$

4.9 Stability Studies

The NLC were packed in glass vials sealed with rubber caps and were kept under fixed temperature and moisture condition (4^0C, 30^0C, 40 ± 2^0C and 70±5% RH) for a period of six months. After stipulated time period the sample were redispersed in distilled water and the stability of NLC was evaluated on the basis of particles size, Entrapment Efficiency of aqueous sample. The results were expressed as mean ± SD (Phatak, et al., 2013;Sánchez-López, et al., 2017).

4.10 Statistical Analysis

The results were expressed as mean values ± SD. One and two way analysis of variance was applied (as applicable) to examine significance of difference for *in-vivo* studies. In all cases, $p < 0.05$ was considered to be significant. Post hoc analysis such as Tukey and Newman-Keuls were also applied based on suitability (Tanno, et al., 2007).

4.11 Fourier Transform Infrared (FT-IR) spectroscopy

The chemical structure of Rifabutin was analyzed by FT-IR spectrophotometer (Schimadzu, Model 8400, Japan) to investigate the possible chemical interactions between the precirol ATO-5. Briefly, the samples of pure precirol ATO-5 and Rifabutin were crushed individually with dry potassium bromide in mortar and pestle followed by compression into a pellet by using pressed pellet technique. The pellet was placed in the FT-IR sample holder and IR spectra, in absorbance mode, was obtained in the spectral region 4,000 to 400 cm^{-1}. Data were collected over 20 scans at 4° resolution.

4.12 Sample Identification through different Parameters:

Sample of rifabutin was identified with the physical parameters such as appearance, odor, melting point, crystallinity, partition coefficient, as per the official procedure. Outcomes of these parameters are discussed in further chapter.