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Formulation and Evaluation of Rifabutin Loaded Nanoparticles Targeting Respiratory Disease

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ABSTRACT

Tuberculosis is a ubiquitous, highly contagious chronic granulomatous communicable bacterial infectious disease caused by *Mycobacterium tuberculosis* and other species of same genera. "Rifabutin" which is useful in the management of tuberculosis. Clinical management of tuberculosis possess serious problem because the efficacy of chemotherapy has been reduced which may be attributed to the degradation of drugs before reaching the target, the low level of cell permeability to drugs, or primary drug resistance. Other reason for the failure of chemotherapy may be the difficulty in achieving adequately high concentration at the infection site, inadequate penetration of drug into macrophages and low level in cells. These problems, which arise with conventional dosage forms of antitubercular drugs, may be overcome by designing and developing a site specific delivery of antitubercular drug using surface modified solid lipid nanoparticles. Hence in the present study, it was attempted to formulate Rifabutin in the form of solid lipid nanoparticle. Solid lipid nanoparticles of Rifabutin were obtained by adaption of lipid dispersion method. Pre formulation studies were performed to check the compatibility of drug and excipient for the preparation of formulation by DSC and no interaction was found. Solubility study, partition coefficient determination, UV analysis, HPLC study, FTIR study were also performed. After the pre formulation studies, Rifabutin loaded solid lipid nanoparticles and mannosylated Rifabutin loaded solid lipid nanoparticles were prepared and a comparison was made between drug entrapment, surface morphology, particle size, PDI and in vitro drug release. Study, hepatotoxicity evaluation and fluorescence microscopy were performed. Hence it can be concluded enhanced alveolar macrophage uptake of Rifabutin loaded mannosylated solid lipid nanoparticles suggest that loading another drug will open new and exciting gateway in the management of tuberculosis.

KEYWORDS: Rifabutin, Tuberculosis, Solid lipid nanoparticle, Antitubercular drug

INTRODUCTION:

TUBERCULOSIS:

Tuberculosis is a universal, profoundly infectious granulomatous transmittable bacterial disease brought about by *Mycobacterium tuberculosis* and different types of same genera. The circumstance has been

exacerbated in view of the presence of various other convoluting factors like multi-drug resistant tuberculosis (MDR TB) and HIV- coinfection. In 1993, the World Health Association proclaimed TB a "worldwide crisis", since just about 33% of the total populations infected with M. tuberculosis[1]. It is evaluated by the World Health Organization (WHO) that in excess of 2 billion individuals on the planet are infected with Mycobacterium tuberculosis (Starke. Jeffrey R, 1997).The introduction of anti-tuberculosis drugs in the 1950s and the development of the various drug regimens meant that by the 1980s there was a 98% chance of cure. Resistant to the most effective drugs; so called multi-drug resistant tuberculosis (Davies Peter D. O., 1999) [2].

Two types of tuberculosis bacilli that affect the human.

- *Mycobacterium tuberculosis* (endemic in man) is transmitted by inhalation of the organism in droplets.
- *Mycobacterium bovis* (endemic in cattle) is transmitted by ingestion of infected milk.

Mycobacterium Tuberculosis

Pathogenic mycobacteria cause diseases of diverse nature and varying severity (Sundaramurthy *et. al.*, 2007) the term mycobacteria is used to designate 3 species of same the genus: *M tuberculosis, M bovis, M Africanism*. Humans are the only reservoir

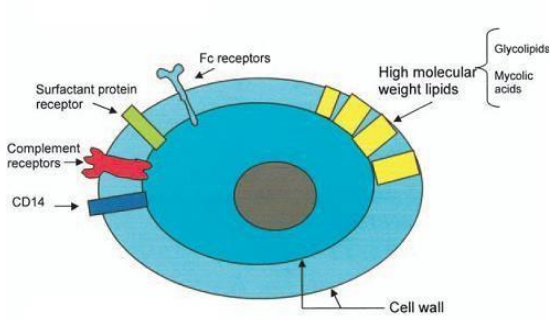

Fig.1.1: Cross section of *Mycobacterium tuberculosis* (Yepes *et. al.*, 2004).

For *Mycobacterium tuberculosis*. The organism is an aerobic, non-motile, non-spore-forming bacillus[3].

Classes of Antitubercular drugs

First line: These drugs have high antitubercular efficacy as well as low toxicity. For eg rifampicin, isoniazid, pyrazinamide

Second line: These drugs have either low antitubercular efficacy or high toxicity or both for eg, Ethionamide, Cycloserine, p-aminosalicylic acid, Clofazimine,

Novel drug delivery system for the treatment of tuberculosis

Drug delivery, which takes into consideration the carrier, the route and the target, has evolved into a strategy of processes or devices designed to enhance the efficacy of therapeutic agents through modified or controlled release. This may involve enhanced bioavailability, improved therapeutic index, or improved patient acceptance or compliance (Flynn 1982) [4].

Solid lipid nanoparticles (SLN's)

Solid lipid nanoparticles (SLN's) are colloidal carrier particles for controlled drug delivery (Hu *et al.*, 2005). SLN's are particles that fall within the size range of 50-1000nm (or1µm). For pharmaceutical applications, all formulation excipients must have Generally Recognized as Safe (GRAS) status. The SLNs possess good tolerability (being derived from physiological lipids), scaling-up feasibility, the ability to incorporate hydrophobic/ hydrophilic drugs and an enhanced stability of incorporated drugs. Thus, SLNs are unique in the sense that they combine the virtues of traditional nanoparticles while eliminating

some of their demerits. (Pandey *et al.*, 2004) [5].

MATERIALS AND METHODS:

Drug common name: Rifabutin
Molecular formula: $C_{46}H_{62}N_4O_{11c}$ NC_4 O_{11}
Molecular weight: 847.02

PREPARATION OF SOLID LIPID NANOPARTICLE:

In this method solid lipid nanoparticles was prepared by solvent injection method. Briefly, Tristearin, soya lecithin, stearylamine and drug (Rifabutin) were dissolved in ethanol at 70˚C and was then rapidly injected through an injection needleinto a stirred aqueous phase maintained at same temperature containing the surfactant,Tween 80 which was previously dissolved in the aqueous phase. The resulting dispersion was then filtered with a paper filter in order to remove any excess lipid. [6]

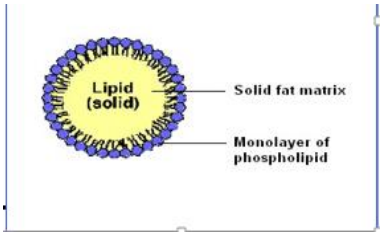

Fig 1.2 solid lipid nanoparticle

PREFORMULATION STUDIES:

The pre formulation studies, which were performed in this project, include identification of drug, solubility analysis, and Partition coefficient and drug compatibility with the lipids.

IDENTIFICATION TESTS

Physical Appearance

The drug (Rifabutin) was obtained as a gift sample from Lupin Pharma Pvt.Ltd, Pune. Physical Appearance noted visually

Melting point

Melting point of Rifabutin was determined by melting point apparatus (Tempo,Mumbai)

Solubility

The sample was qualitatively tested for its solubility in various solvents. It was determined by shaking 10 mg of drug sample in 10 ml of solvent (i.e., water, methanol, ethanol, ether, chloroform, benzene etc.) in small test tubes and noted down the time require to disappear the sample completely.

Determination of λ_{max}

Accurately weighed 10 mg of Rifabutin was dissolved in 100 ml of methanol in a 100 ml volumetric flask. Then, 1 ml of this stock solution was pipetted into a 10 ml volumetric flask and volume made up to the mark with distilled water. The resulting solution was scanned between 200 -400 nm using Cintra 10 GB UV-visible spectrophotometer. The same procedure was followed for determining the λ_{max} in PBS (pH7.4) and sodium acetate buffer of pH 4.0 except methanol was replaced with the respective solutions.

Infrared Spectroscopy

Drug sample was vacuum dried for 12 hours before IR studies. Drug (5mg) was mixed with potassium bromide (100mg) and compressed into pellets. The IR spectrum was taken in Department of Sophisticated analytical instrument facility in Panjab University, Chandigarh.

Partition coefficient

The partition behavior of drug was examined in n-octanol: water, n-octanol: PBS (7.4) system. It was determined by taking 5 mg of drug in two separating funnelsone containing 10 ml portions of n-octanol and 10 ml water and the other containing, 10 ml of n-octanol and 10 ml of PBS (pH 7.4) respectively. The separating funnels were shaken for 2 hr in a wrist action shaker for equilibration. Two phases were separated and the amount of the drug in aqueous phase was analyzed spectrophotometrically at 275 nm after appropriate dilution. The partition coefficient of the drug was calculated by using the following formula

$$\text{The partition coefficient, K} = \frac{\text{Amount of drug in organic layer}}{\text{Amount of drug in aqueous layer}}$$

PREPARATION OF STANDARD CURVE OF RIFABUTIN IN DIFFERENT SOLUTIONS:

Preparation of Standard curve of Rifabutin in Methanol

Accurately weighed 10 mg of Rifabutin was dissolved in methanol and volume was made up the mark in a 100 ml volumetric flask. This resulted in 100 □g/ml stock solution. The aliquots of 0.2 ml, 0.4 ml, … up to 2 ml of stock solution were transferred into a series of 10 ml volumetric flasks and volume was made up to the mark with double distilled water. The solutions were filtered through Whatman filter paper and filtrate analyzed at $\square_{max}$ 275 nm using Cintra 10 UV Visible Spectrophotometer. The standard curve was plotted between absorbance andconcentration.

Preparation of Standard curve of Rifabutin Phosphate Buffer Solution pH 7.4

Preparation of PBS pH 7.4

Disodium hydrogen orthophosphate (1.38g), potassium dihydrogen orthophosphate (0.19 g) and sodium chloride (8.0g) were added to about 100 ml of distilled water and the volume.Was made up to 1000 ml with distilled water. The pH of solution was adjusted to 7.4 immediately before use with 0.1 N hydrochloride acid or 0.1 NaOH as required.

Preparation of standard curve in PBS pH 7.4

For the preparation of standard curve in PBS pH 7.4, all dilutions and, measurements were made same as discussed above except the methanol was replaced with phosphate buffer saline pH 7.4

Preparation of Standard curve of Rifabutin sodium acetate Buffer Solution pH 4.0

Preparation of sodium acetate buffer solution pH 4.0

Glacial acetic acid (2.78 ml) and 50% w/v of sodium hydroxide (1ml) were mixed and the volume was made up 100 ml with distilled water, the pH of solution was adjusted to 4.0 immediately before use with acetic acid.

Preparation of standard curve in sodium acetate buffer solution (pH 4.0)

For the preparation of standard curve in sodium acetate buffer solution (pH 4.0) all dilutions and measurements were made same as discussed above except the methanol was replaced with sodium acetate buffer solution (pH 4.0). The absorbance of different drug solutions was taken at $\square_{max}$ 275 nm against a reagent blank. The standard curve was plotted between absorbance and concentration.

Preparation of Standard curve of Rifabutin in Serum using HPLC method

The blood of the mice was collected by cardiac puncture and collected in the bottle containing heparin sodium (100 IU/ml in saline) as anticoagulant. The blood sample was centrifuged at 5000 rpm for 10 minutes. Supernatant was collected and then it was deproteinized with acetonitrile for half an hour to precipitate the proteins. The precipitated proteins were separated by centrifugation at 5000 rpm for 10 minute and supernatant was collected. Collected supernatant (serum) was filtered through 0.45-µm-membrane filter. From this serum, seven sets of serum samples with varying drug concentration of 100 to 2500 ng/ml were prepared and filtered. The filtrate (20□l) was injected into a reverse phase C-18 column (HPLC column) and the eluents were monitored at 275 nm. The peak areas of Rifabutin were recorded (the regression of plasma/serum concentration of the drug over its peak area was calculated using the least squares method of analysis). The HPLC equipment consisted of RP C-18 column, variable wavelength programmable UV/vis detector SPD-10A VP, mobile phase used for estimation of Rifabutin was 0.05 M potassium dihydrogen phosphate and 0.05 M sodium acetate solution (pH adjusted to 4.0 with acetic acid): acetonitrile (53:47). The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 ml/min. The column temperature was Maintained at 25^{o}C.the volume of each injection loop was 20□l.

Drug Compatibility Studies with Selected Lipids

Drug compatibility with Tristearin, phosphatidyl choline (PC) and stearylaminewas studied. Solution of Rifabutin (8□g/ml) was prepared in methanol. Then, accurately weighed lipid (10 mg) was transferred separately into 10 ml volumetric flasks containing drug solution. The flasks were shaken for 3 hours and absorbance was measured for each solution using Cintra-10 UV Spectrophotometer against respective blank solution. The absorbance data are recorded.

OPTIMIZATION

Drug concentration, lipid concentration, emulsifier concentration and process variables i.e. stirring speed, stirring time and sonication time. The optimization was done on the basis of particle size, poly dispersity index and drug loading efficient.

OPTIMIZATION OF FORMULATION VARIABLES

Optimization of lipid/lecithin ratio

For optimization of lipids ratio, the SLNs formulations were prepared with varying ratio of two lipids i.e. Tristearin and soya lecithin in the different ratios (viz. 1:0.5, 1:1, 1:1.5, 1:2 %w/w) keeping other parameters constant.Optimization was done on the basis of average particle size and poly dispersity index (PDI) of SLNs, which were determined using Malvern Zetasizer.

Optimization of drug/lipid ratio

For optimization of drug/lipid ratio the SLNs formulation LL_2 was selected and different SLNs formulations were prepared with ratio 5:100, 10:100, 15:100, 20:100 % w/w of drug and lipids (Tristearin and PC), keeping the other parameters constant .Optimization was done on the basis of average particle size of SLNs and percent drug entrapment. The drug entrapment efficiency was determined by mini column centrifugation technique.

Optimization of surfactant concentration

For optimization of concentration of tween 80, formulation D_2 LL_2 was selected. Keeping the other parameters constant, SLNs formulations were prepared using different concentration of tween 80 in

aqueous medium. The effects of emulsifier concentration on the particle size and entrapment efficiency are reported.

Optimization of Lipid/Stearylamine (SA) ratio

Formulation D_2 LL_2 T_2 was selected for the optimization keeping other parameters as constants. Lipid: Stearylamine were taken in the different ratios 100:0.5, 100:1.0, 100:1.5 and 100:2. Results are recorded.

A. Optimization of Process Variables

Process variables i.e. stirring speed, stirring time and sonication affect the formulation very much. Formulation D_2 LL_2 T_2 SA_2 was selected as the optimized formulation.

Optimization of stirring speed

Stirring speed of the stirrer was varied from 1000 to 4000 rpm for SLNs preparation using the same formula of optimized formulation parameters and particle size and percent drug entrapment were determined and are shown.

Optimization of stirring time

For the optimization of stirring time D_2 LL_2 T_2 SA_2 R_3 was selected while other process variables were kept constant. The SLNs dispersion was prepared by stirring for different time periods (viz. 15, 30, 45 and 60 min.). Further the particle size and percent drug entrapment were determined and are shown.

Optimization of sonication time

Formulation D_2 LL_2 T_2 SA_2 R_3 t_2 was selected for the optimization of sonication time. SLNs formulations were prepared with varying sonication time period i.e. 1, 2, 3, 4. Average particle size was determined using Zetasizer 3000 HS (Malvern, UK) and percent drug entrapment was also determined.

Optimized Parameter

The optimization studies were performed on the SLNs formulation D_2 LL_2 T_2 SA_2 R_3 t_2 X and the parameters used for the preparation of this optimized formulation are shown.

CHARACTERIZATION OF SOLID LIPID NANOPARTICLES

A. Particle size determination:

For the determination of average particle size and polydispersity index of the solid lipid nanoparticles Photon Correlation Spectroscopy using a Zetasizer (DTS Ver. 4.10, Malvern Instruments, and England) was used. The sample of dispersions was diluted with 1: 9 v/v-deionized water.

B. Surface charge measurement

The zeta potential of the nanoparticles was determined by laser Doppler anemometry using a Malvern Zetasizer also called Doppler Electrophoretic Light Scatter Analyzer. The instrument is a laser-based multiple angle particle electrophoresis analyzer.

C. Particle Morphology (TEM)

Transmission electron microscope (TEM) was used as a visualizing aid for particle morphology. The sample (10µL) was placed on the grid and allowed to stand at room temperature for 90 sec. All samples were examined under a transmission electron microscope (Philips Morgagni 268, Eindhoven, Netherlands) at an acceleration voltage of 100 kV, and photomicrographs were taken at suitable magnification, which was shown.

D. Surface Morphology (SEM)

Surface morphology was determined by Scanning Electron Microscope (SEM) at AIIMS, New Delhi. The samples for SEM were prepared by lightly sprinkling the SLN powder on a double adhesive tape, which was stuck on an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å by

using a sputter coater. All samples were examined under a scanning electron microscope (LEO 435 VP, Eindhoven Netherlands) at an acceleration voltage of 30 kV, and photomicrographs were taken at suitable magnifications, which are shown.

RESULTS

SOLUBILITY PROFILE OF RIFABUTIN

Table: 1 Solubility of Profile of Rifabutin

S.NO	SOLVENT	SOLUBILITY
1	Water	Insoluble
2	PBS	Slightly soluble
3	Sodium acetate buffer (pH 4.0)	Slightly soluble
4	Methanol	Freely soluble
5	Ethanol	Sparingly soluble
6	Ether	Insoluble
7	Chloroform	Freely soluble

Physical Appearance	Supplied sample of Rifabutin was red-violet, crystalline, odorless, hygroscopic powders
Melting point	Melting point was found to be 153□2 °C.
Determination of λ_{max}	The λ_{max} was found to be 275 nm

UV scan of Rifabutin in methanol

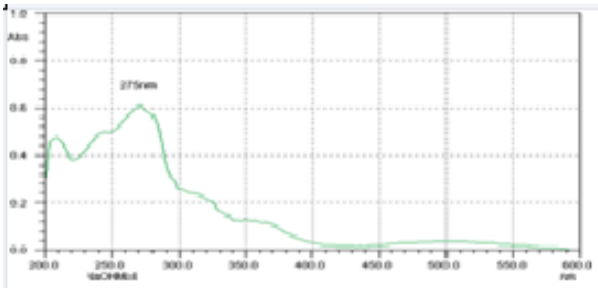

Fig 1.3 UV Scan of Rifabutin in methanol

UV Scan of Rifabutin in PBS (pH7.4)

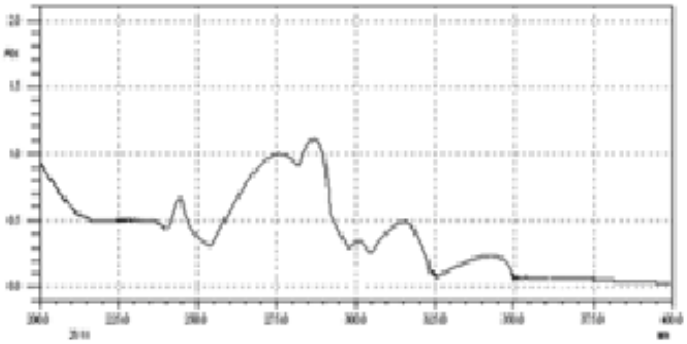

Fig 1.4 UV Scan of Rifabutin in PBS (pH 7.4)

UV scan of Rifabutin in sodium acetate buffer (pH 4.0)

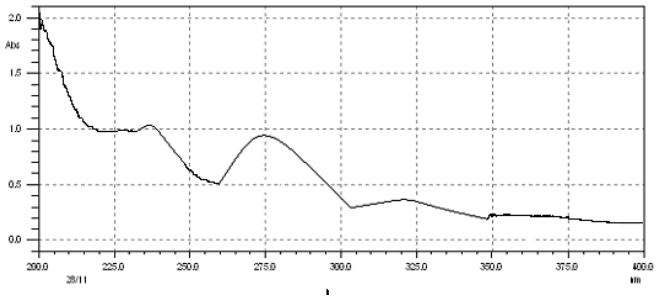

Fig: 1.5 UV scan of Rifabutin in sodium acetate buffer

Infrared Spectroscopy

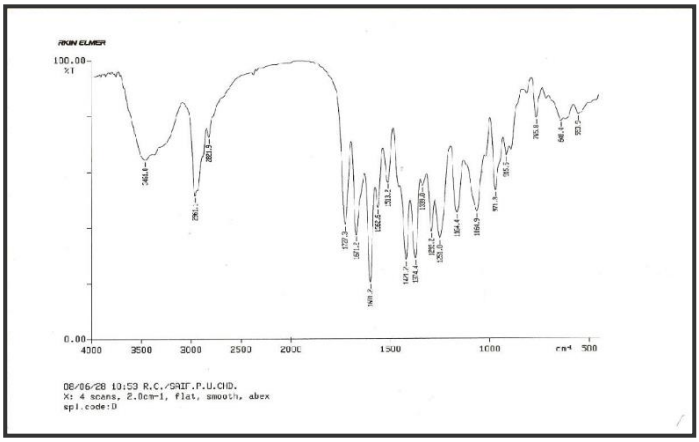

Fig: 1.6 IR spectroscopy of Rifabutin

Partition Coefficient

Table: 2 Evaluation of Partition Coefficient

S.no	Medium	Partition coefficient (n-octanol/aq. Phase)
1	n-Octanol : Water	3.1
2	n-Octanol : PBS pH (7.4)	2.9

Standard curve of Rifabutin in Methanol (□max= 275 nm)

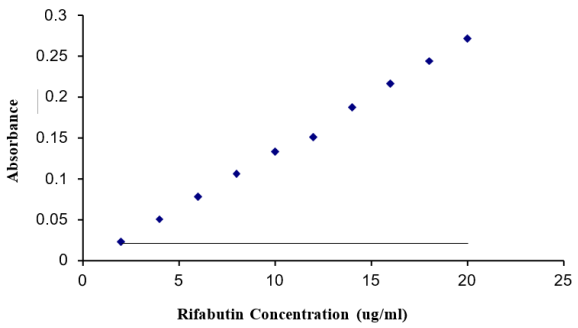

Fig: 1.7 Standard curve of Rifabutin in methanol

PBS (pH 7.4) □□max 275 nm

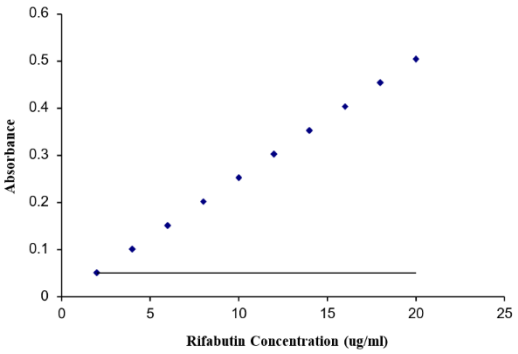

Fig: 1.8 standard curve of Rifabutin in PBS

Buffer solution (pH 4.0) at λ_{max}275 nm

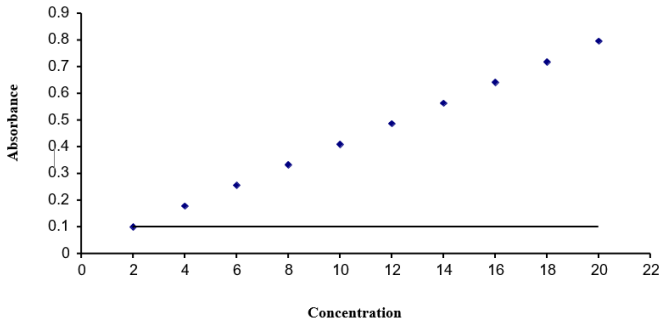

Fig: 1.9 standard curve OF Rifabutin in Buffer Solution

Calibration curve of Rifabutin in serum using HPLC method

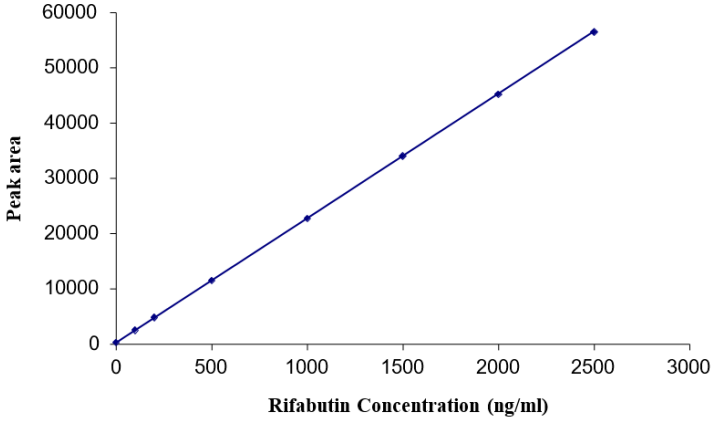

Fig: 1.10 Calibration curve of Rifabutin

Drug compatibilities studies with selected lipids

Table: 3 Drug Compatibilities Studies

S. No.	Composition	ɔrption maxima(λ_{max}) nm	Absorbance
1.	Rifabutin (8µg/ml)	275	0.2990
2.	Rifabutin+ Tristearin	275	0.2950
3.	Rifabutin + Lecithin	275	0.2705
4.	Rifabutin+ Stearylamine	275	0.2497

Optimization of best Formulation:

Optimized Parameters of optimized formulation i.e.D_2 LL_2 T_2 SA_2 R_3t_2X

Table: 4 Parameters of Optimized Formulation

Parameter	**Optimized Value**
Tristearin: Lecithin ratio Drug: Lipid ratio	100:100
Surfactant conc.	
Stirring Time	10:100 mg
Stirring Speed Sonication Time	
	1% wt./v

	30 min 3000 rpm 2 min

In *vitro* drug release in phosphate buffer (pH 7.4) from SLNs formulations

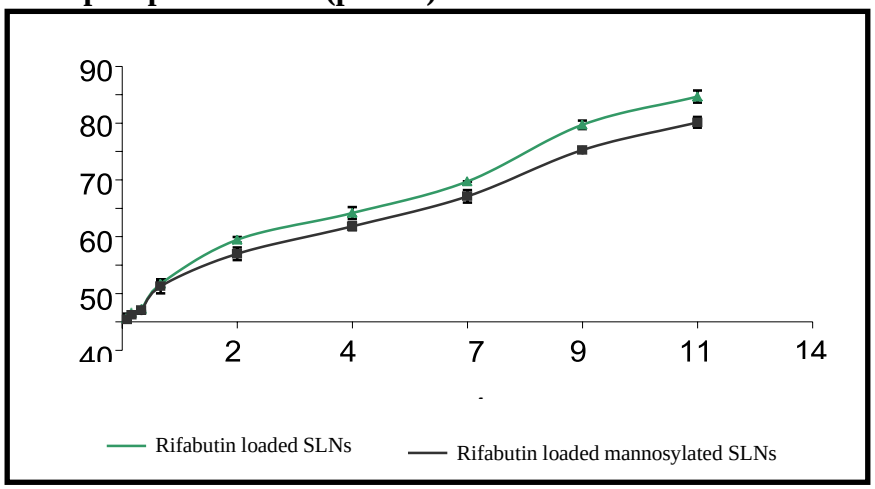

Fig: 1.11 In *vitro* Drug Release in Phosphate buffer

In vitro drug release in sodium acetate buffer (pH 4.0) from SLNs formulation

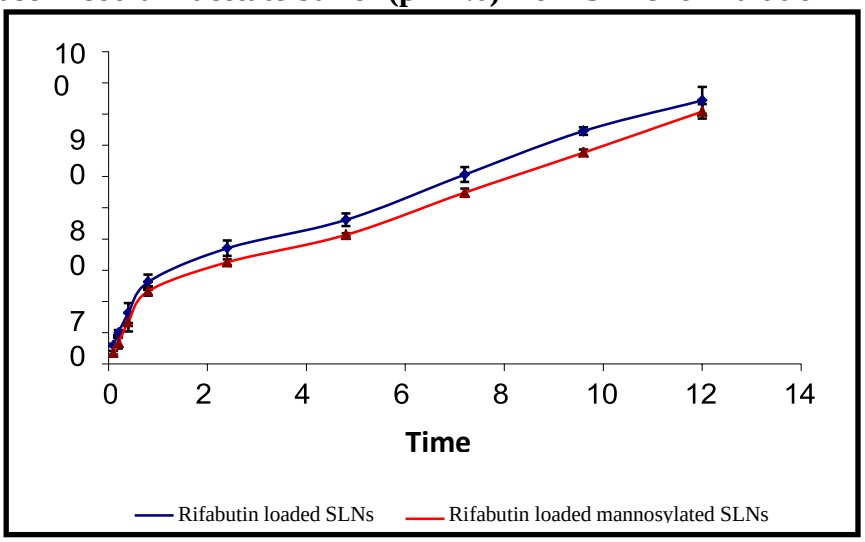

Fig: 1.12 In *vitro* Drug Release in sodium acetate buffer

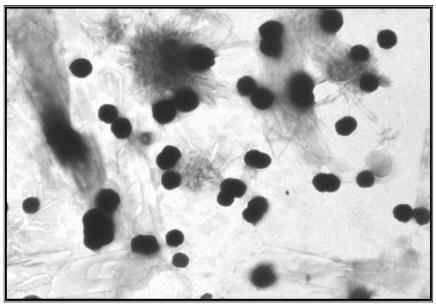

Fig: 1.13 TEM of Rifabutin loaded SLNs

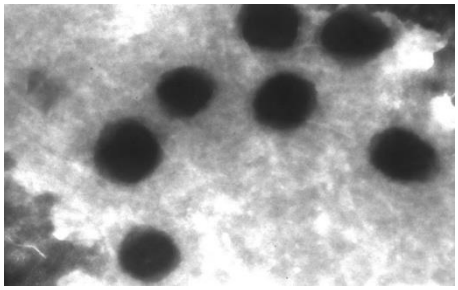

Fig: 1.14 TEM-Rifabutin loaded Mannosylated SLNs

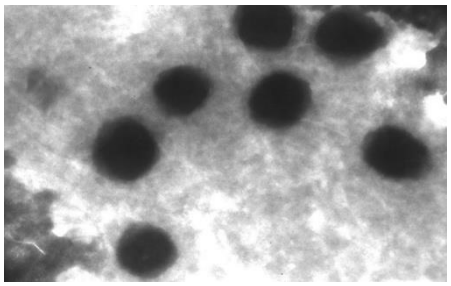

Fig: 1.15 SEM-Rifabutin loaded SLNs

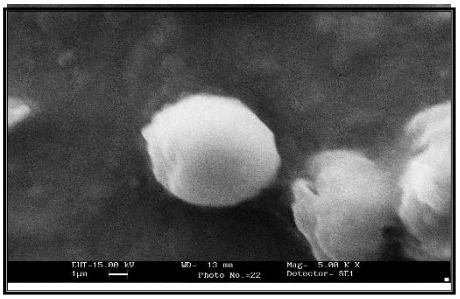

Fig: 1.16 SEM of Rifabutin-loaded mannosylated SLNs

DISCUSSION

For this study, Rifabutin loaded SLNs and Rifabutin loaded mannosylated SLNs was prepared and characterized bySEM, TEM and IR spectroscopy. The optimized formulation has the lipid: lecithin ratio of100:100 having particle size of 250□3.0 nm and a PDI of 0.439. Drug: lipid ratio was selected to be 10:100 which has the particle size of 242□3.8 nm and percent drugentrapment of 89.4□0.4. The optimized surfactant concentration was found to be 1%, which has particle size and percent drug entrapment of 237□2.5 and 89.6□0.6 respectively. Lipid/stearylamine ratio was found to be 100:10 resulting in SLNs

of particle size and percent drug entrapment was found to be 234□7.2 and 88.9□2.4 respectively. 3000 rpm was found to be the optimum speed and 30 min. was the optimumtime having particle size of 230□3.5 and percent drug entrapment of 87.5□2.4. Theoptimized sonication time was 2 min. resulting in SLNs of particle size of 251□5.1 and percent drug entrapment of 87.8□1.2. The optimized formulation was coded as $D_2LL_2T_2SA_2R_3t_1X$.

SUMMARY AND CONCLUSION:

A Comparison study was performed between Rifabutin loaded SLNs and Rifabutin loaded Mannosylated SLNs. A comparison was made between drug entrapment, surface morphology, particle size, PDI and *in vitro* drug release. Particle size of Rifabutin loaded SLN formulation $D_2LL_2T_2SA_2R_3t_1X$ was smaller than Rifabutin loaded mannosylated SLNs .The result was found to be Rifabutin loaded Mannosylated solid Lipid Nanoparticles have better action than Rifabutin loaded nanoparticle.

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