Current Trends in Biotechnology and Pharmacy
Vol. 12 (2) 169-176 April 2018, ISSN 0973-8916 (Print), 2230-7303 (Online)

In-Vivo Evaluation of Rifampicin Loaded Nanospheres: Biodistribution and *Mycobacterium* Screening Studies

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Abstract:

Rifampicin PLGA nanospheres are formulated with a specific goal in order to decrease the dose, adverse effects and to enhance targeted drug delivery. Rifampicin nanospheres were prepared and evaluated by emulsion solvent evaporation method. *In vivo* bio distribution studies reveal that there was a long term accumulation of rifampicin nanospheres in the lungs over other organs. The increase in C_{max} values confirmed that inhalable PLGA nanospheres are suitable for targeting and providing sustained release of anti-tubercular drugs to lungs. So inhalation is a selected administration route of Rifampicin PLGA nanospheres. The *in vivo* screening of *M. tuberculosis* showed good activity as well as its activity against multidrug-resistant *M. tuberculosis* and against *M. tuberculosis* isolates in a potentially latent state, makes Rifampicin PLGA nanospheres as an attractive drug dosage form for the therapy of tuberculosis. It can be concluded that there is a significant potential for effective oral delivery as well as nasal delivery of the Nanospheres for the treatment of tuberculosis.

Keywords: Rifampicin Nanospheres, *M. tuberculosis, In vivo* Bio distribution studies.

Introduction

Tuberculosis is a contagious disease that transmits through air by the bacterium *Mycobacterium tuberculosis* (MTB) (1). Despite the pathogen being pulmonary targeted, it's pathogenicity spreads over entire body. In addition, tuberculosis displays a vibrant range from non infectious to hazardous ailment (2, 3).

Development of specific delivery system with sustained release of drug can able to maintain the adequate therapeutic concentration in the site of action (4). Use of bio decomposable and biocompatible polymers to develop nanospheres is remarkable achievement in controlled drug delivery system.

Poly lactic co glycolic acid (PLGA) polymer its co-polymers have achieved a perspective in preparing an array of delivery systems incorporated with various drugs for sustained release, being their biodegradable and biocompatible characters with minimum toxicity (5) in particular as anti tubercular drug (ATD) carrier (6).

Current research was aimed to investigate pre clinically for efficacy release of pulmonary targeted ATD rifampicin from PLGA nano carrier in comparison with intravenous route therapy of the same using the conventional formulation.

Materials and Methods

Materials

Rifampicin and polyvinyl alcohol (PVA) were obtained from Sigma Chemical Co. Poly (lactide-co-glycoside) was purchased from Boehringer Ingelheim, Germany. Remaining chemicals used in this study are of analytical grade.

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Formulation of Rifampicin PLGA Nanoparticle by Emulsion Solvent Evaporation Method : Different variants of rifampicin stacked nanoparticles (NPs) were prepared by changing the formulation variables (like polymer and surfactant) and process variables (like sonication time). In the formulations, 100 mg of rifampicin was dissolved with various concentrations of PLGA and surfactant in 300 ml ethanol. Then the organic phase was emulsified by adding different concentrations of PVA using ultrasonicator for 35 seconds. The emulsion was included drop wise into beaker containing different concentration of PVA solution which acts as continuous phase and mixture was maintained for continuous stirring. The emulsion was left on gentle stirring for 3 hrs to allow for solvent evaporation. Then, suspended nanoparticles were collected by ultracentrifugation at 30,000 rounds per minutes (RPM) for 15 min at 3° C. The NPs were washed three times with cold double-deionized water and then freeze-dried for 38 h (7).

Pharmacokinetic Studies : Rodents like Wistar albino rat of male gender (160-180g) were selected for investigation. Rats were resided in polypropylene cages in a proper aerated room under atmospheric circumstances of 22±2°C and 45-65% relative humidity, with on and off light with equal duration in a day. Selected rats were adjusted to laboratory conditions one week prior to the date of experimentation. Standard diet food and water were given at sufficient levels. Rats were fasted overnight prior to the day of experiment with a provision of water.

On the next day, Rifampicin nanospheres were administered to rats (n= 6) by intravenous route through tail vein at the dose of 2.4 mg/rat. Rats are bled at time periods 5,15,30 min,1,2,4,6,8,12 and 24 h following administration. After centrifugation, the plasma was divided and frozen at -20°C for further studies (7).

Bio-distribution studies : Male Wistar rats (n=6) weighing 160–180g were used for the bio distribution studies. The experimental proposal was accepted and performed as per the guidelines of Institutional Animal Ethical Committee (IAEC) (MIP/IAEC/2015-16/M1/07) of the Institute. Nanospheres at a dose of 25mg/rat were administered once by inhalational route. About required dose of drug loaded nanospheres were charged and nebulized for 30 sec using an in house apparatus to obtain inhaled dose of 25mg/rat. Before dosing, the rats were trained for 3 days to accept or restraint the application of an infant inhalation mask attached to our in-house apparatus.

After inhalation, rats were bled at different time intervals selected for Bio distribution studies. After blood collection, animals were sacrificed by using CO_2 euthanasia and organs like lungs, liver and kidney were collected. The organ weights were recorded. Lungs were kept in saline prepared with phosphate buffer at a slight acidic pH and stored at -20ºC until analysis. The collected organs are sliced and homogenized at 6000 RPM for 20 min. Centrifugation was done to the collected tissue samples at 4000 RPM for 10 min and the collected supernatant was analyzed by HPLC.

Collection of bronchi -alveolar ravage : From the sacrificed rats lungs were isolated in conjunction with trachea by dissecting thoracic region. The lungs were frequently lavaged with ice cold phosphate buffer saline (PBS) (with 0.5M EDTA) through cannulated trachea. Broncho alveolar fluids were pooled, made centrifuge and the collected macrophages were numbered and stored at -20ºC till the analysis.

The analysis of drug was done by HPLC and the concentration of drug was obtained from calibration graphs. Inspecting the data visually, maximum plasma concentration (C_{max}) and time to achieve it time to maximum plasma concentration (T_{max}) were determined.

The plasma concentration values were transformed logarithmically and by applying linear regression $T_{1/2}$ was estimated. The plasma concentration versus time curve (zero moment) and the first moment curve area under the moment curve (AUMC) were estimated. The area under the curve (AUC), area under the concentration-

time curve from time zero to time of last sample intake (AUC_{0b} t) and area under the moment curve-time curve from time zero to time of last sample intake ($AUMC_{0b}$ t) were calculated as per the trapezoid rule. The first moment was calculated as concentration times time (Cp x t). The AUMC is the area under the (Cp x t) versus time curve. The AUC determines the bioavailability of the drug for the given same dose in the formulation. Total calculations were done by software Phoenix WinNonlin non-compartmental analysis program.

Sample preparation for analysis

Blood sample: The blood samples collected at respective time intervals during pharmacokinetic and bio distribution studies were taken in heparinised micro-centrifuges. Blood samples were centrifuged at 4000 rpm for 10 min to separate plasma and it was maintained at -20ºC until analysis.

Aliquots of 150µl of plasma were mixed with methanol (300µl) as de-proteinizing agent and the obtained dispersal is whirl pooled around 2 min. The samples were centrifuged at 15000 RPM for 10min at 4°C and the supernatant is collected.

Rifampicin was extracted with 3 ml of chloroform-butanol (70:30%v/v) and 3 ml portions of chloroform-butanol (70:30% v/v) and vortexed for about 1 min followed by centrifuging at 4000 RPM of 10 min duration. The superficial layer was decanted and the process was repeated for in triplicate and superficial liquid was collected. The collected supernatants were diluted and analyzed by HPLC.

Tissue sample : Tissue homogenates (20% w/v) with aqueous medium were prepared in cold 150M KCL. Supernatant liquid collected from homogenates by centrifugation at 15000 RPM for 10 min at 4°C was kept aside for further studies. Then, 300µl of the methanol was admixed to 150µl of the clear homogenates and the dispersal was vortexed for 2 min. The samples were then centrifuged at 15,000 RPM for about 10 min at 4 ºC. An equal volume of water is added to the obtained supernatant. The samples were further filtered using 0.2µm nylon filters and were instilled to the HPLC system (8-11).

Bio-analytical HPLC method : The collected serum and tissue samples were analyzed by HPLC (Analytical technologies Ltd) comprising C-18 column & UV detector. The mobile phase consists of Triethanolamine acetate: acetonitrile (97: 3% v/v) eluted by isocratic method and detected at 262 nm, analysed at 30ºC by injecting 20µl by maintaining a flow rate of 0.9ml/min. A wash method program which increased the % methanol was included at the end of drug elution to ensure washout of all interfering exciepents. Spectral purity analysis of the drugs peak over a range of 200–400 nm was performed. The accuracy and precision of the developed method for determination of drug was comparable to the isocratic methods described in United State Pharmacopoeia (USP).

Data Analysis

The area under the total plasma concentration time curve from zero time to infinity was calculated by equation $AUC_{0-\infty}$ = area under the plasma concentration-time curve extrapolated to infinity

$$AUC_{0-\infty} = AUC_{0-t} + C_t/K_e$$

Where C_t is the rifampicin concentration observed at last time and K_e is the apparent elimination rate constant obtained from the terminal slope of the individual plasma concentration (12-18). Durations of serum drug concentration following inhalational route were analyzed by software Phoenix Win Nonlin non-compartmental analysis program of version 5.1.

Mycobacterial infection study in rat

MTB H37Rv ATCC 27294, a strain sensitive to all the standard antimycobacterial agents, was used for all animal infection in the experiments. Bacterial cultures were prepared as described previously published article (8). Wistar rats were infected via the respiratory route to obtain low-grade bacillary lung infection (100 bacilli) using a modified Madison aerosol chamber (9). Bacterial lung loads were estimated to determine suitable infection conditions for drug efficacy experiments. After infection, the animals were housed for the duration of the study in a bio-safety level 3 facilities. By using microbial enumeration,

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dependent variable, the number of animals required, per treatment group was three in experiments for drug evaluation. The course of mycobacterial infection was monitored by enumeration of colony forming units (CFU) from excised lungs at 1, 2, 3, 4, 6, and 12 weeks of post infection.

Statistical analysis : All the experiments were performed in triplicate. Results were collected as mean and standard deviation (mean ± SD). Significance in difference was measured with p value as p d" 0.5.

Results

The mean bio distribution pharmacokinetic study parameters of F13 rifampicin nanospheres formulation administered through Intravenous (IV) and Inhalation are summarized in the Table 1, Figure 1 & 2. The C-max, T-max and clearance of F13 through Inhalation route were 42.34 ìg/mL, 14 hr and 0.82(ml/hr) respectively, similarly those of IV route were 20.46 ìg/mL,14 hr and 3.44 respectively.

In short term study (Table-3),the potency of rifampicin nanospheres at 25 mg/kg body weight was compared with those of rifampicin pure drug. rifampicin nanospheres slightly more active than pure drug rifampicin. Long-term treatment (Table-3) with rifampicin nanospheres at 25 mg/kg Shown statistically very significant when compared to negative control in the lungs and spleen ($p<0.05$). The results elucidated that there was a significant decrease in colony forming unit (CFU) of lungs and spleen in all treated groups during both period in contrast to negative control group. After 18 days of treatment, rifampicin nanospheres minimized the bacterial content by 0.54 $\log_{10}$ CFU. The activity of rifampicin nanospheres and rifampicin pure drug in the lungs and spleen was statistically not significant (p = ns) when compared with negative control after 18 days of treatment. After 42days of treatment, rifampicin nanospheres minimized the bacterial content 4.62$\log_{10}$ CFU. (9.68 14.30-9.68 log 10 CFU). The efficacy of rifampicin nanospheres in the lungs and spleen differs statistically from those of rifampicin pure drug ($P > 0.001$) after 42days of treatment. The results are shown in (Table 2&3 and Figure no 3).

Discussion

By comparing the C_{max} results between IV and Inhalation administration of Rifampicin Nanospheres it shows that more concentration of drug was accumulated in the lungs while

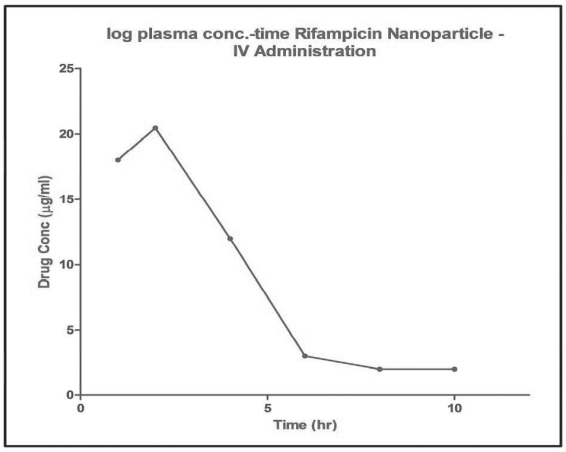

Fig. 1 : XY plot for Rifampicin Nanoparticle –IV administration

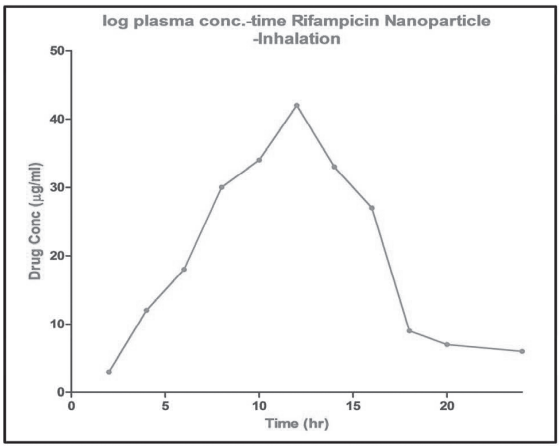

Fig. 2: XY plot for Rifampicin Nanoparticle – Inhalation administration

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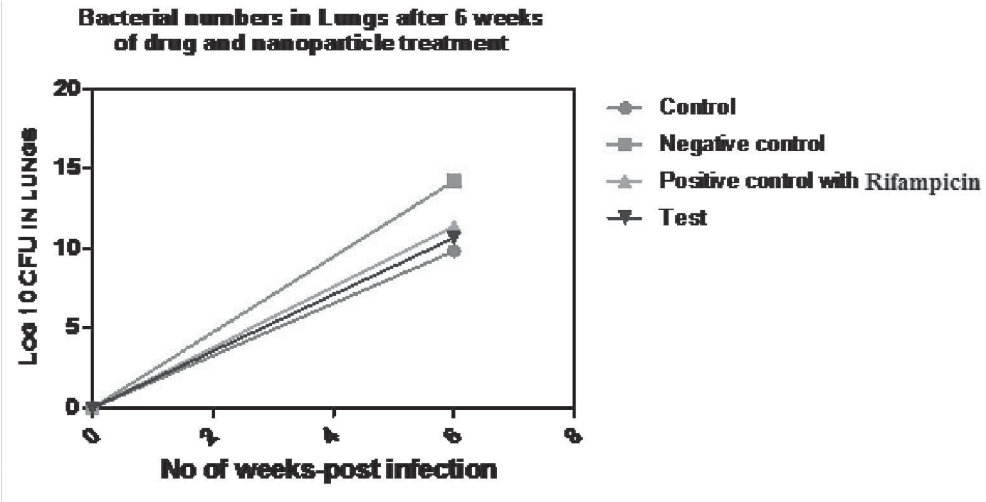

Fig. 3: Determination of Bacterial numbers in lungs after 6 weeks of treatment

Table 1: Bio distribution studies of Rifampicin Nanospheres (F13) – IV & Inhalation administration

Tissue	T+(hr)	C_{max}(µg/ml)	$AUC_{0-"}$ (µg/ml/hr)	V_d (ml)	Clearance (ml/hr)
IV Administration					
Lungs	1	20.46±1.05	802.3±5.29	244.8±3.95	3.44±0.31
Liver	2	24.98±1.17	1120.16±8.46	184.61±2.10	2.82±0.56
Kidney	3	22.02±0.94	690.39±5.93	218.43±42	3.02±0.14
Inhalation					
Lungs	14	42.34±2.46	1996±8.32	136.33±2.48	0.82±0.04
Liver	20	10.84±1.8	484±4.26	489±5.73	2.84±0.19
Kidney	24	4.32±0.86	118±3.16	756.6±6.38	2.34±0.23

Table 2: Growth of *M.tuberculosis* in culture medium and their control in different conditions

Compound	Concentration	Log_{10}CFU±SD (per ml)	% growth of microorganism compared with controls
Only culture	-	11.62±0.22	100
Pure Rifampicin	25 mg/kg	9.40±0.12**	78.40
Rifampicin NP	Equivalent to 25 mg/kg	6.42±0.26***	68.40

Values are expressed as mean ± SD. n=3; Values are statistically significant at p<0.05; Significant-p<0.05;** Very significant -p<0.01;***-p<0.001; **ns**-non-significant;

Table 3: *M.tuberculosis* number in lungs and spleen of albino rat

Treatment batch	*M.tuberculosis* number (Log_{10}CFU±SD) after different treatment schedules			
	18days		42 days	
	Lungs	Spleen	Lungs	Spleen
Control group	6.40 ± 0.12	2.16 ± 0.12	9.42 ± 0.42	4.12 ± 0.10
Negative control	7.42± 0.32^{a**}	2.74 ± 0.10^{a**}	14.30± 0.54^{a***}	5.68 ± 0.22^{a***}
Positive control with Rifampicin 25 mg/kg	7.40 ± 0.20$^{b\ ns}$	2.54 ± 0.14$^{b\ ns}$	10.40 ± 0.30^{b***}	3.64 ± 0.24^{b***}
Test group with Rifampicin Nanospheres Equivalent to 25 mg/kg	7.82 ± 0.22$^{b\ ns}$	2.48 ± 0.10^{bns}	9.68 ± 0.22^{b***}	2.28 ± 0.22^{b***}

Values are expressed as mean ± SD. n=3; Values are statistically significant at $p<0.05$; Significant-**-$p<0.01$;Very Significant-***-$p<0.001$; **ns**-non-significant; **a**-Group compared to control; **b**-Groups compared to negative control;

administering the formulation through Inhalation. From the T_{max} comparison data between IV and Inhalation administration it shows the sustainability time of rifampicin nanospheres in lungs i.e., it confirms the sustained action of rifampicin nanospheres in lungs. By comparing the $AUC_{0-á}$ from Table 1, it concludes that maximum concentration of drug was present in lungs through inhalation than any other organ. The organ clearance ratio of drug from lungs through inhalation was less than the IV administration, which confirms the sustained release of rifampicin from nanospheres in the lungs. From the above pharmacokinetic distribution data it shows that rifampicin nanospheres shows more accumulation of drug in lungs through inhalation administration than IV, which indicates nanospheres is having targeted and sustained release of drug results in lungs. By this it can be confirmed that inhalable nanospheres are suitable for targeting with negligible toxicity and providing sustained release of anti-tubercular drugs especially rifampicin in lungs. The result showed the rifampicin nanospheres leads to maximum deposition of drug in lungs through inhalation which leads to maintain high therapeutic concentration by improving good pulmonary tuberculosis chemotherapy.

In vivo mycobacterium screening studies show that on long term therapy rifampicin nanospheres shows better control of growth of microorganism i.e. CFU when compared to short term therapy i.e. for 18 days after administration. After 6 weeks of treatment the bacterial counts in the lungs were reduced to very low numbers in all treatment groups (range, 14.30 to 9.68 log10 CFU Rifampicin Nanospheres Vs untreated controls), as was the case for the spleens ($p<0.001$). In summary, its good activity *in vivo* models, as well as its activity against multidrug-resistant *MTB*and against *MTB* isolates in a potentially latent state, makes Rifampicin Nanospheres an attractive drug dosage form for the therapy of tuberculosis. These data indicate that there is significant potential for effective inhalational delivery of Rifampicin Nanospheres for the treatment of tuberculosis.

Conclusion

In vivo bio distribution studies show that nanospheres form is the best formulation for Rifampicin, Nanospheres accumulates maximum

dose in the lungs than other organs over prolonged period of time. The plasma levels are more for inhalable PLGA Nanospheres and are suitable for targeting and providing sustained release of anti-tubercular drugs to lungs. So inhalation can be selected as administration route of Rifampicin PLGA Nanospheres. From the *in vivo* screening of *M.tuberculosis*, it shows good activity *in vivo* models, as well as its activity against multidrug-resistant *MTB*and against *MTB*isolates in a potentially latent state, makes Rifampicin PLGA Nanospheres an attractive drug dosage form for the therapy of tuberculosis. These data indicate that there is significant potential for effective intravenous as well as nasal delivery of Nanospheres for the treatment of tuberculosis**.**

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