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Studies on *invivo* and biodistribution perspectives of solid lipid nanoparticles encompass isoniazid in animal model

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ABSTRACT

TB is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary TB). The disease is spread in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. *Invivo* Biodistribution studies which was carried for the best formulation shows that, FS3 Isoniazid Solid lipid nanoparticle accumulates maximum dose of Isoniazid in the lungs than other organs over prolonged period of time by enhanced Cmax which confirmed that inhalable SLN are suitable for targeting and providing sustained release of anti-tubercular drugs to lungs. So Inhalation is an selected administration route of Isoniazid SLN. From the invivo screening of *M.tuberculosis*, its good activity *in vivo* models, as well as its activity against multidrug-resistant M. tuberculosis and against M. tuberculosis isolates in a potentially latent state, makes Isoniazid SLN an attractive drug dosage form for the therapy of tuberculosis. These data indicate that there is significant potential for effective oral delivery of Isoniazid SLN for the treatment of tuberculosis.

Keywords: *Mycobacterium tuberculosis*, Invivo studies, SLN Isoniazid nanoparticles

INTRODUCTION

Tuberculosis (TB) is a disease caused by bacteria that are spread from person to person through the air. TB usually affects the lungs, but it can also affect other parts of the body, such as the brain, the kidneys, or the spine. In most cases, TB is treatable and curable; however, persons with TB can die if they do not get proper treatment. Multidrug-resistant TB (MDR TB) is caused by an organism that is resistant to at least Isoniazid and Rifampin, the two most potent TB drugs. These drugs are used to treat all persons with TB disease [1-6].

In general, a relatively small proportion of people infected with *Mycobacterium tuberculosis* will develop TB disease; however, the probability of developing TB is much higher among people infected with the human immune deficiency virus (HIV). TB is also more common among men than women, and affects mostly adults in the economically productive age groups. Without treatment, mortality rates are high. In studies of the natural history of the disease among sputum smear-positive and HIV-negative cases of pulmonary TB, around 70% died within 10 years; among culture-positive (but smear-negative) cases, 20% died within 10 years Tuberculosis (TB) has been a leading cause of death since time immemorial and it continues to cause immense human misery even today [7, 8].

Tuberculosis is chronic granulomatous disease in major health problem in developing countries about 1/3rd of world population infected by mycobacterium tuberculosis it is apprehended with that unless urgent action is taken less than 15 million people worldwide including 4 million in India will die from tuberculosis in 1st decade of 21st century according to WHO as the ICMR 2001 estimate 40% adults in India are effected nearly 2 million people develops active disease every year about 0.5 million people die from it. The World Health Organization (WHO) *Global Tuberculosis Report 2012* provides the latest information and analysis about the tuberculosis (TB) epidemic and progress in TB

TYPES OF TUBERCULOSIS

Tuberculosis is divided into three clinically important categories

- Primary TB
- Secondary Reactivated TB
- Disseminated TB

Globally, 40% of TB patients had a documented HIV test result and 79% of those living with HIV were provided with co-trimoxazole preventive therapy in 2011. Interventions to detect TB promptly and to prevent TB among people living with HIV, that are usually the responsibility of HIV programmes and general primary health-care services, include regular screening for TB and isoniazid preventive therapy (IPT) for those without active TB.

Bio-Distribution Studies of SLN- Isoniazid

For in vivo pharmacokinetic studies, Male Wistar rats weighing 160–180 gm were used. The protocol was duly approved by the Institutional Animal Ethics Committee (IAEC).

Route of administration: Inhalation

A group of male Wistar rats (n=6) received a single dose of 100 mg of SLN by inhalational route. About 500 mg of drug loaded SLN were charged & aerosolized by 30 actuation/30 sec using an in house apparatus to obtain inhaled dose of 100mg/animal (here with the help of blower the formulation is administered). Before dosing the rats were trained for 30 days to accept restraint & application of an infant inhalation mask attached to our in-house apparatus.

Sample collection

Collection of blood

After inhalation, the pharmacokinetic study was done by collecting blood sample at different time intervals viz., before dosing, 10 min after dosing & then for 1, 2, 4, 8, 12, 24 hr time periods by tail incision.

After blood collection animals were administrated with ketamine-xylazine for deep anesthesia, there thoracic cavity is opened and tissues of interest is collected eg. Lungs, Liver, Kidney were excised bottled dry, weighed & kept in triple distilled water at -20-20ºC. The collected organs are sliced and homogenized at 6000 rpm for 20 min. The tissue fluids are collected and centrifuged at 4000 rpm for 10 min and finally the supernatant is collected and analysed by HPLC.

Collection of broncho -alveolar lavage

After sacrifice, thoracic cavity is opened; lungs intact with trachea are excised. The trachea was cannulated & lungs were repeatedly lavaged with chilled PBS (containing 0.5 M EDTA) broncho alveolar fluids were pooled, centrifuged and macrophages obtained were counted and kept at -20ºC for further analysis.

Sample preparation for analysis

The blood samples were collected from tail incision (0.5ml) was taken in heparinised micro-centrifuge tubes containing heparin equivalent to 50µl/ml of blood at different time intervals. Plasma was separated by centrifuging the blood samples at 4000 rpm for 10 min at 4ºC. Blood serum was collected and kept at -20ºC until analysis.

To 150 ml aliquots of plasma, 300 ml of de-protenizing agent (methanol) was added and the dispersion is vortexed for 2 min. The samples were centrifuged at 15,000 rpm for 10 min at 4ºC and supernatant is collected [9-18].

Isoniazid was extracted using 3 ml portions of chloroform-butanol (70:30 %v/v) and vortexed for 1 min followed by centrifuging at 4000 rpm for 10 min. supernatant was decanted , process was repeated for 3 times & supernatants was pooled. The collected supernatants were diluted and analysed by HPLC.

Tissue sample preparation

20% (w/v) aqueous tissue homogenates were prepared in cold 150M KCl. The homogenates were centrifuged at 15,000 rpm for10 min at 4ºC

and the clear supernatant thus obtained was used further. To 150µL aliquot of the clear tissue homogenates, 300µL of the methanol was added and the dispersion was vortexed for 2 min. The samples were then centrifuged at 15,000 rpm for 10 min at 4ºC. The supernatant was collected and an equal volume of water was added. The samples were then filtered (0.20µm nylon filters) and were injected into the HPLC system.

Bio-analytical HPLC method

The collected serum samples were analysed by HPLC (Analytical technologies Ltd) comprising C-18 column & UV detector. The mobile phase consists of Triethanolamine acetate: acetonitrile (97: 3 %v/v) at 263 nm by isocratic elution method. The mobile phase was delivered at a flow rate of 0.9 ml/min and The injection volume was 20µL and the analysis was performed at 30ºC.

A wash program which increased the % methanol was included at the end of Isoniazid elution to ensure washout of all interfering excipients. Spectral purity analysis of the Isoniazid peak over a range of 200–400 nm was performed. The accuracy and precision of the developed method for determination of Isoniazid was comparable to the isocratic methods described for Isoniazid in USP.

Invivo screening of Mycobacterium tuberculosis

M. tuberculosis isolation and culture

The *M.tuberculosis* strain was grown, by collecting the sputum of the TB patient and inoculated in liquid medium containing 0.05% Tween 80 to mid-log phase, aliquoted, and frozen at 80°C until use in further in vitro assay. Aliquots of frozen bacteria were used MIC assay. The cultures were grown in 10-ml volumes in tubes with screwcap of 25X150 mm, at 37°C with rapid stirring for 7 to 10 days until they reached mid-log phase. The virulent M. tuberculosis strain was grown to mid-log phase in liquid medium containing 0.05% Tween 80 and frozen in aliquots at 70°C until needed. This isolate has been used as the standard strain in tests of the activities of drugs and formulation in animal models.

Micro dilution (Minimum inhibitory concentration) MIC plate assay

A method was used to determine the MICs by a microdilution plate assay by using *M. tuberculosis*. Isoniazid was dissolved in sterile, double-distilled water at a stock concentration of 200 µg/ml. Isoniazid SLN was dissolved in 100% dimethyl sulfoxide (DMSO) to a stock concentration of 50 µg/ml. A 1:2 dilution series of both compounds was made in a separate microtiter plate by using the same diluents. The interior round-bottom microtiter assay plate was seeded with bacterial suspension. Two microliters of each drug and formulation i.e SLN nanoparticle was transferred to the assay plate wells containing bacteria. The final concentrations of Isoniazid in the wells ranged from 5.0 to 3.0 µg/ml; the final concentrations of Isoniazid SLN ranged from 2.0 µg/ml to 4.0 µg/ml. The assay plates were incubated at 37°C for at least 21 days and were observed every 3 to 4 days to evaluate changes in growth. Inhibition of growth was determined both by visual examination [19].

Drug preparation for *in-vivo* models

Isoniazid pure drug was dissolved in 50% Dimethyl sulfoxide (DMSO), with subsequent dilution in sterile water prior to administration. The final concentration of DMSO in the drug preparation was 5%. Isoniazid SLN, dose was taken equivalent to 100-mg/kg dose based on acute toxicity studies. For the preparation of the lower and higher doses (50 and 300 mg/kg was taken), the amount of drug was adjusted.

In-vivo screen

The mice were infected via a low-dose aerosol exposure to *M. tuberculosis* Erdman in a Middlebrook aerosol generation device, and the short-course mouse model was performed i.e., short exposure of mice to *M.tuberculosis*. One day post infection, three mice were killed to verify the uptake of 50 to 100 (colony forming unit) CFU of bacteria per mouse. Following infection, the mice were randomly divided into 4 treatment groups. Negative control mice remained untreated. Positive control mice received Isoniazid (at 25 mg/kg of body weight). Test group received Isoniazid SLN formulated (equivalent to 25 mg/kg). Each treatment group consisted of six mice. Treatment was started 18 days post infection and continued for nine consecutive days. Three infected mice were killed at the start of treatment as pretreatment controls. Drugs and dosage form were administered daily by oral. The treatment was continued to next 6 weeks and the

lungs and spleen samples are tested at the end of the treatment. [75, 76]

Enumeration of viable *M. tuberculosis* in mouse organs

The mice were anaesthetized by Chloroform inhalation. The spleens and left lung lobes were aseptically removed and disrupted in a tissue homogenizer. The number of viable organisms was determined by serial dilution of the homogenates on high speed homogenizer (CAT, Germany). The plates were incubated at 37°C in ambient air for 6 weeks prior to the counting of viable *M. tuberculosis* colonies (CFU). After long-term treatment, the entire volume of each organ homogenate was plated to determine the total number of culturable mycobacteria per organ [20].

STATISTICAL ANALYSIS

The viable counts were converted to logarithms, which were then evaluated by a one-way analysis of variance, followed by a multiple comparison analysis of variance by a one-way test (Sigma Stat software program). Differences were considered significant at the 95% level of confidence.

RESULTS AND DISCUSSION

Bio-Distribution Pharmacokinetics Studies

Table 1: Bio distribution studies of SLN- Isoniazid (FS3) – IV Administration

Drug/route/origin	Tmax(hr)	Cmax(µg/ml)	AUC $_{0-\infty}$ (µg/ml/hr)	V_d (ml)	Clearance (ml/hr)
Lungs	2	18.24+3.4	848+54.4	244.80+8.26	3.40+ 0.04
Liver	3	24.98 +4.0	1006 +124.4	184.6+20.4	2.94+0.50
Kidney	5	22.02+2.42	680+20.90	218.4+12.6	3.22+0.46

Table 2: Bio distribution studies of SLN- Isoniazid (FS3) – Inhalation Administration

Drug/route/origin	Tmax(hr)	Cmax(µg/ml)	AUC $_{0-\infty}$ (µg/ml/hr)	V_d(ml)	Clearance (ml/hr)
Inhalation Lungs	12	36.82±4.2	1800±109.6	136.33±6.94	1.2±0.22
Liver	18	11.64±0.98	486±17.89	489±23.62	2.84±0.84
Kidney	22	5.24±0.10	218±11.29	756.6±88.5	2.34±0.52

*Standard deviation (n=3)

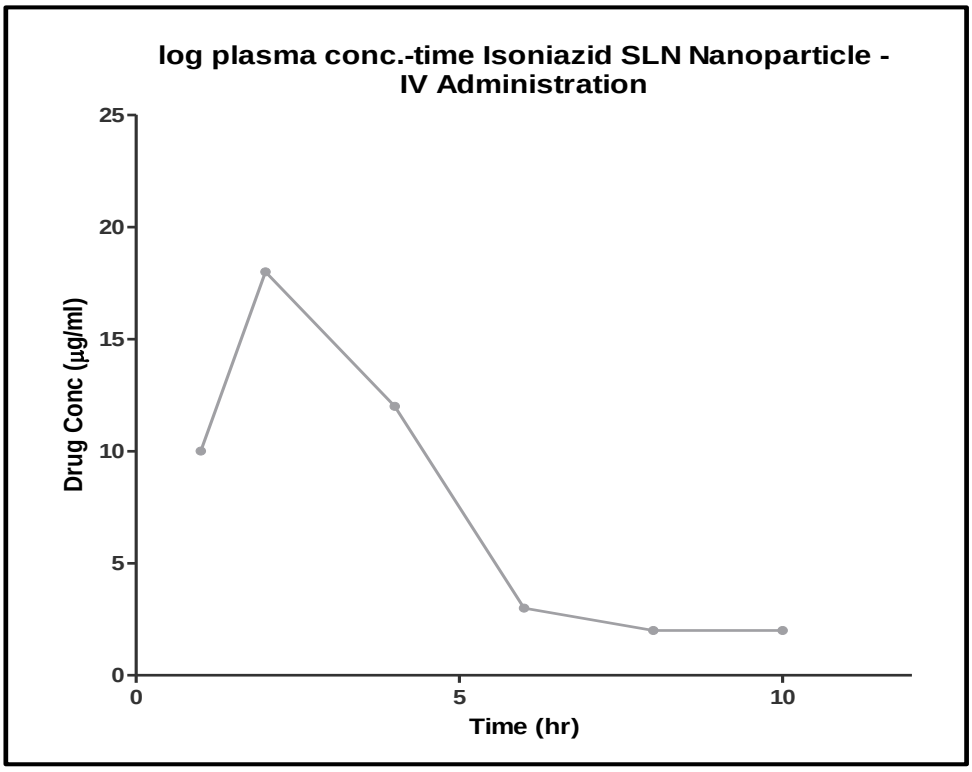

Figure 1: XY plot for Isoniazid SLN Nanoparticle –IV administration

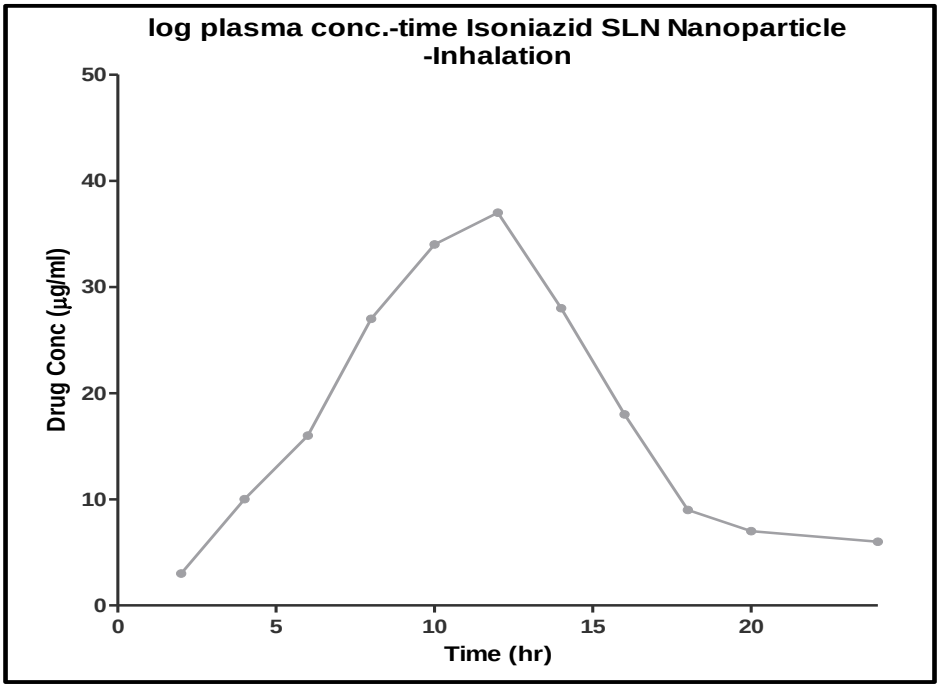

Figure 2: XY plot for Isoniazid SLN Nanoparticle –Inhalation administration

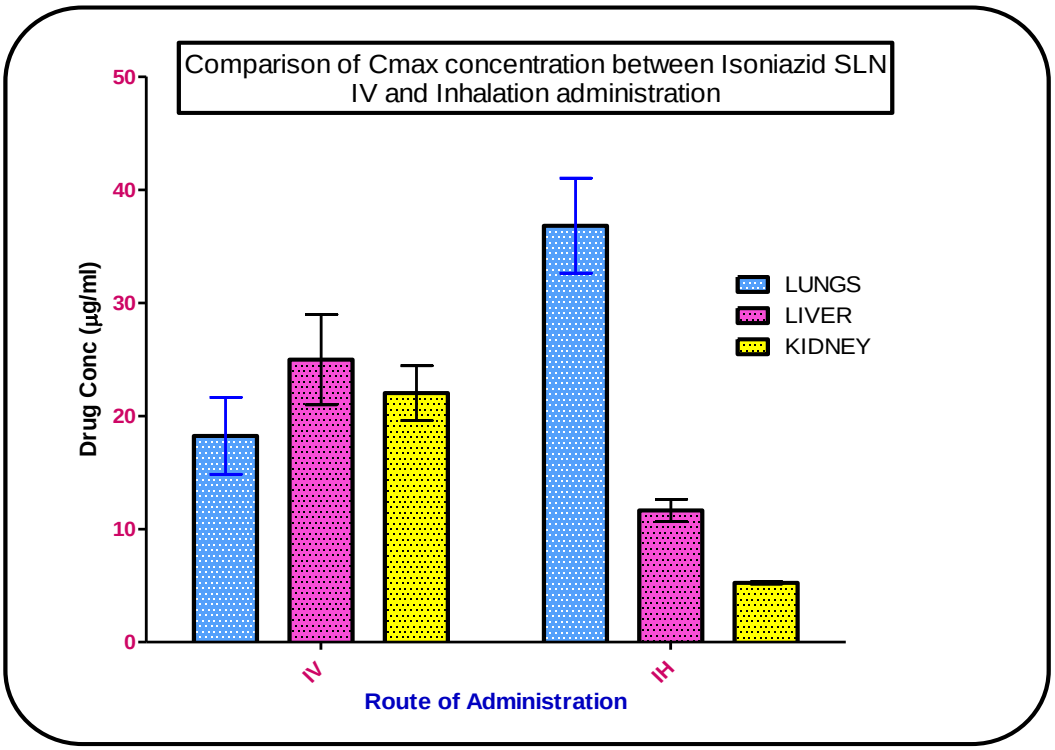

Figure 3: Comparison of Cmax concentration between Isoniazid SLN IV and Inhalation administration

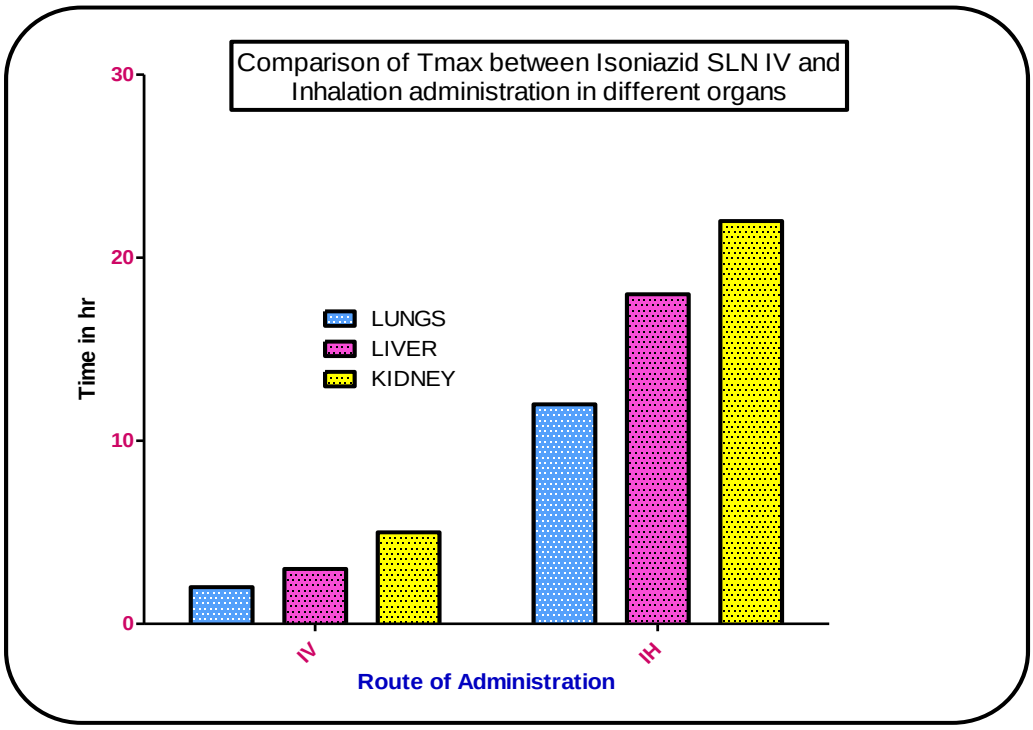

Figure 4: Comparison of Tmax between Isoniazid SLN IV and Inhalation administration in different organs

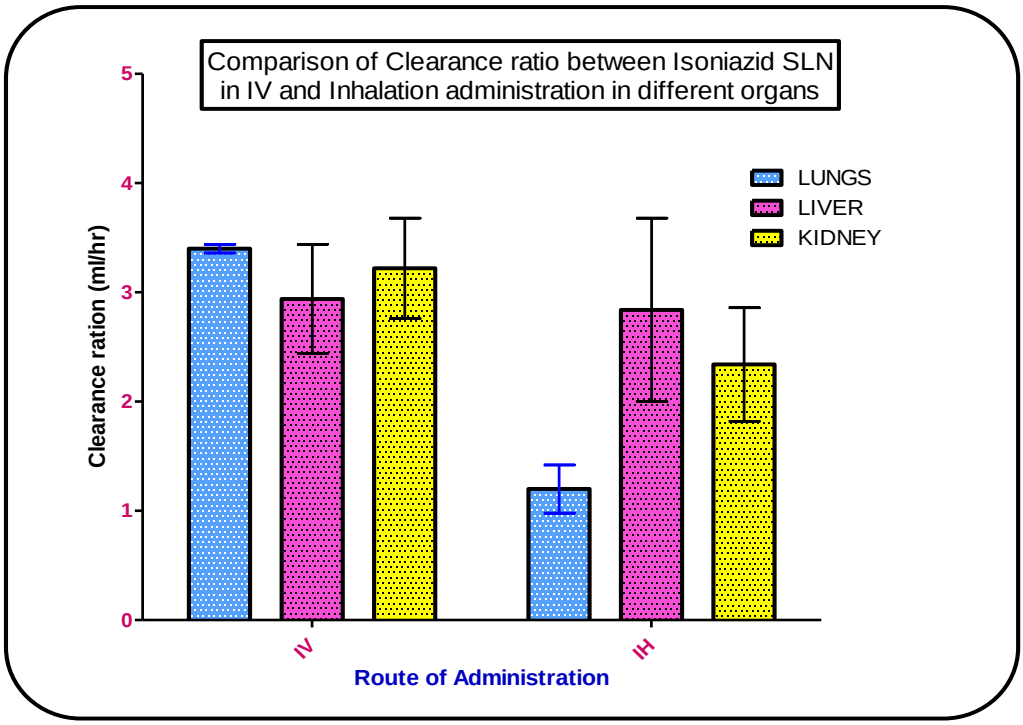

Figure 5: Comparison of Clearance ratio between Isoniazid SLN

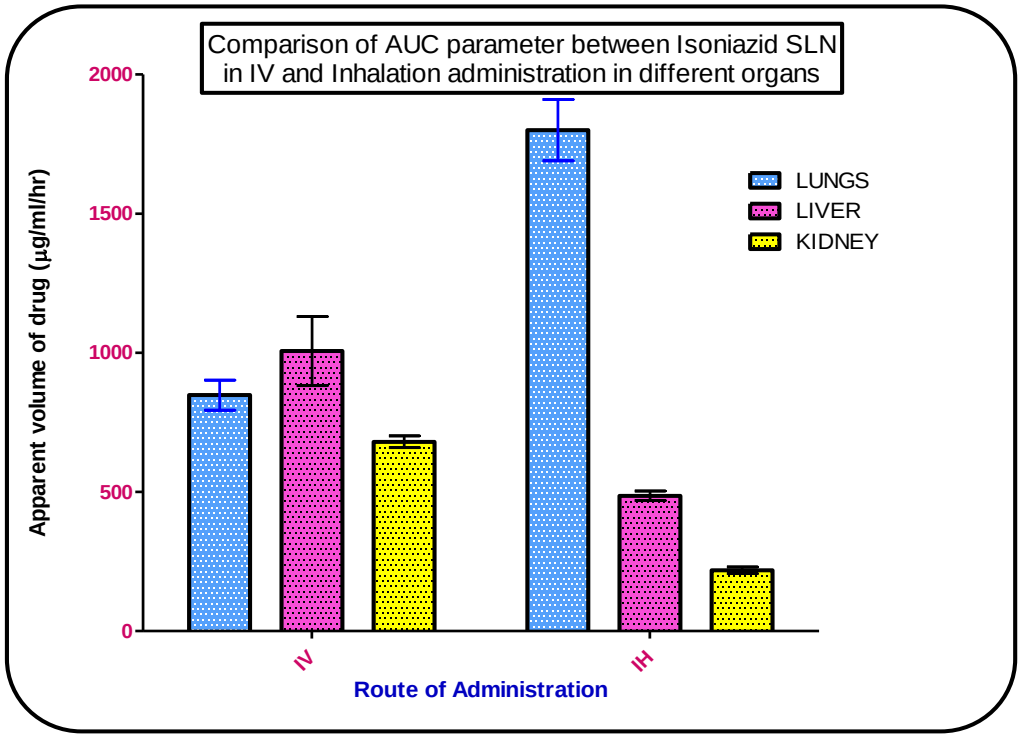

Figure 6: Comparison of AUC parameter between Isoniazid SLN

The mean Biodistribution pharmacokinetic parameters of FS3 SLN- isoniazid formulation administered through IV and Inhalation. The C_{max}, T_{max} and Clearance of FS3 formulation through Inhalation and IV was 36.82 µg/mL, 12hr, 1.2 and 18.24 µg/mL,12 hr,3.40 respectively.

By comparing the C_{max} results between IV and Inhalation administration of Isoniazid SLN it shows that more concentration of drug was accumulated in

the lungs while administering the formulation through Inhalation.

From the T_{max} comparison data between IV and Inhalation administration it shows the sustainability time of Isoniazid SLN in lungs i.e., it confirms the sustained action of Isoniazid SLN in lungs.

By comparing the $AUC_{0-\alpha}$ form above tables, it concludes that maximum concentration of drug was present in Lungs through inhalation than any other organ. And the organ clearance ratio of drug from lungs through inhalation was less than the IV administration, which confirms the sustained release of Isoniazid from SLN in the lungs.

Form the above pharmacokinetic distribution data it shows that Isoniazid SLN shows more accumulation of drug in lungs through inhalation administration than IV, which indicates SLN is having targeted and sustained release of drug results in lungs.

By this it can be confirmed that inhalable SLN are suitable for targeting with negligible toxicity and providing sustained release of anti-tubercular drugs especially Isoniazid in lungs.

The results of this report shows the Isoniazid SLN leads to maximum deposition of drug in lungs through inhalation which leads to maintain high therapeutic concentration by improving good pulmonary tuberculosis chemotherapy.

Invivo Mycobacterium screening studies

The *Invivo* studies revealed that the Isoniazid SLN was tested *in-vitro* against a broad panel of multidrug resistant clinical isolates like *M.tuberculosis* and was found to be highly active against all isolates (MIC < 1 g/ml). The activity of Isoniazid SLN against *M. tuberculosis* was also assessed grown under conditions of oxygen depletion. Isoniazid SLN showed significant activity when compared to pure drug. In a short-course mouse infection model, the efficacy of Isoniazid SLN at 25 mg/kg of body weight after nine oral treatments was compared with those of isoniazid pure drug. Isoniazid SLN, dose equivalent to 25 mg/kg was slightly more active than pure drug isoniazid at 25 mg/kg. Long-term treatment with Isoniazid SLN at 25 mg/kg reduced the bacterial load most effectively in the lungs and spleen. No significant differences in activity between Isoniazid SLN and the other single drug treatments tested could be observed. The results show that all drug treatments reduced the numbers of CFU in the lungs and spleens significantly at every time point compared with the numbers in the untreated controls (P> 0.001).

After 18 days of treatment, Isoniazid SLN reduced the bacterial loads in the lungs by 0.46 log10 CFU (6.54 versus 6.08 log10 CFU for the untreated controls). The activity of Isoniazid SLN in the lungs and spleen was statistically not that much different from those of isoniazid pure drug (P < 0.05) after 18 days of treatment.

After 6 weeks of treatment, Isoniazid SLN reduced the bacterial loads in the lungs by 3.54 log10 CFU (10.68 versus 14.62 log10 CFU for the untreated controls). The activity of Isoniazid SLN in the lungs and spleen was statistically different from those of isoniazid pure drug (P > 0.05) after 6 weeks of treatment.

It shows that on long term therapy Isoniazid SLN shows better control of growth of microorganism i.e. colony forming unit (CFU) when compared to short term therapy i.e. for 18 days after inhibition. After 6 weeks of treatment the bacterial counts in the lungs were reduced to very low numbers in all treatment groups (range, 14.62 to 10.08 log10 CFU Isoniazid SLN Vs untreated controls), as was the case for the spleens.

There is no complete eradication of the bacterial count in any of the mice after 12 weeks of treatment with single compounds as well as the formulation. No statistically significant differences in activity between isoniazid SLN and the pure drug treatments could be observed for the spleens or the lungs (P > 0.05).

In summary, its good activity *in vivo* models, as well as its activity against multidrug-resistant M. tuberculosis and against M. tuberculosis isolates in a potentially latent state, makes Isoniazid SLN an attractive drug dosage form for the therapy of tuberculosis. These data indicate that there is significant potential for effective oral delivery of Isoniazid SLN for the treatment of tuberculosis.

Table 3: Growth of *M.tuberculosis* in culture medium and control in different condition

Compound	Conc. µg/ml	Log_{10}CFU±SD (per ml)	% growth of microorganism compared with controls
Only culture	-	10.92±0.24	100
Pure Isoniazid	25 mg/kg	8.24±0.24	74.24
Isoniazid SLN	Equivalent to 25 mg/kg	7.84±0.24	71.17

Table 4: ***M.tuberculosis*** **numbers in lungs and spleen of albino mice - drug and formulation treatment for 18 days**

Treatment batch	Log_{10}CFU±SD	
	Lungs	Spleen
Control groups	5.42 ± 0.22	2.26 ± 0.12
Negative control	6.54± 0.34	2.80 ± 0.10
Positive control with Isoniazid 25 mg/kg	6.34 ± 0.20	2.60 ± 0.14
Test group with Isoniazid SLN Equivalent to 25 mg/kg	6.08 ± 0.14	2.52 ± 0.16

Table 5: *M.tuberculosis* numbers in lungs and spleen of albino mice - drug and formulation treatment for 6 weeks

Treatment batch	Log_{10}CFU±SD	
	Lungs	Spleen
Control groups	9.84 ± 0.42	4.32 ± 0.16
Negative control	14.22± 0.54	5.78 ± 0.20
Positive control with Isoniazid 25 mg/kg	11.40 ± 0.30	3.66 ± 0.22
Test group with Isoniazid SLN Equivalent to 25mg/kg	10.68 ± 0.22	3.08 ± 0.26

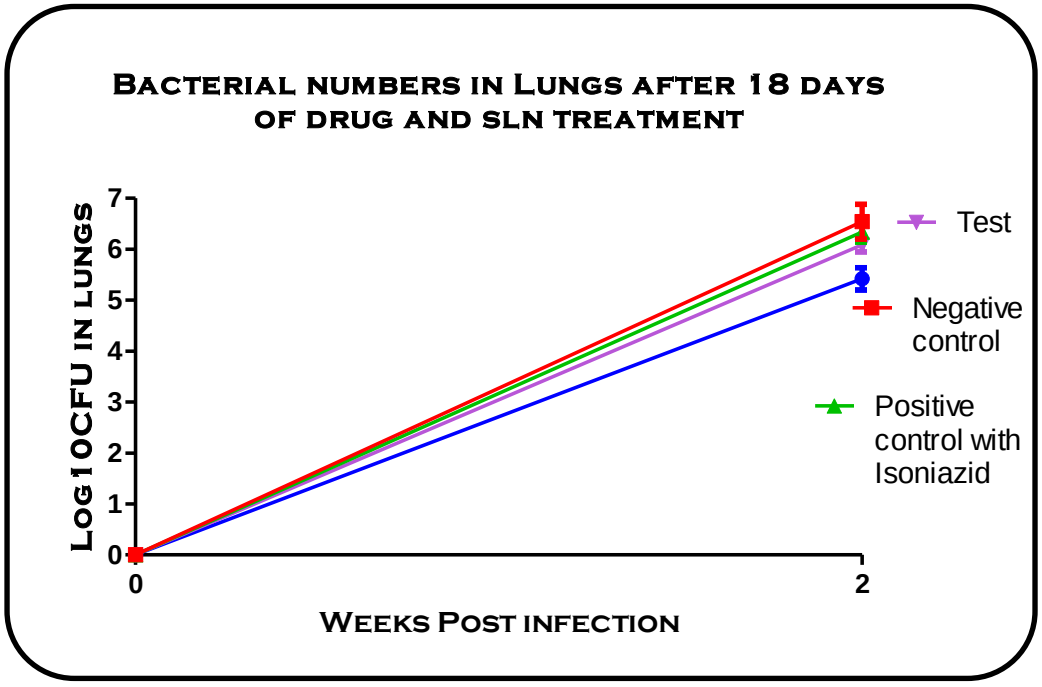

Figure 7: Determination of Bacterial numbers in lungs after 18 days of treatment

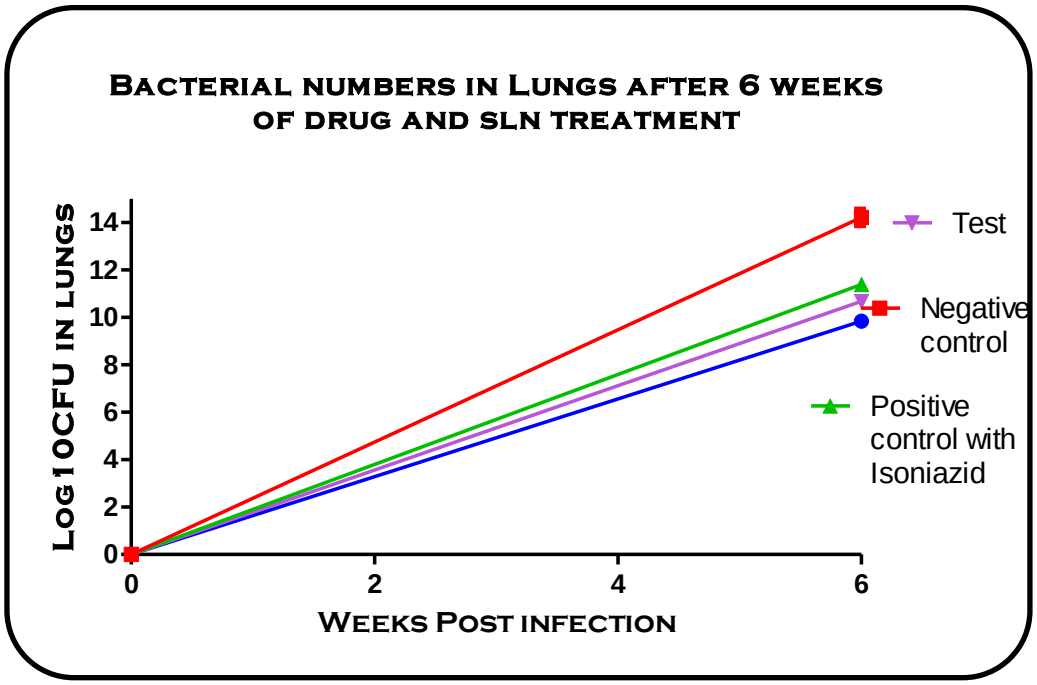

Figure 8: Determination of Bacterial numbers in lungs after 6 weeks of treatment

CONCLUSION

In this research work, it has been concluded that Inhalation is the best choice of administration path for SLN containing antitubercular drugs, which concluded that it was ease to target the Isoniazid drug in the form of SLN will leads to effective control of Lung Tuberculosis with less dose, dosage regimen and enhanced bioavailable dose due to targetability and sustainability of isoniazid from dosage form, It has good activity *in vivo* models, as well as its activity against multidrug-resistant M. tuberculosis and against M. tuberculosis isolates in a potentially latent state, makes Isoniazid SLN an attractive drug dosage form for the therapy of tuberculosis.

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