Lung-Targeted Solid Lipid Nanoparticles for Enhanced Pulmonary Delivery of Anti-Tubercular Drugs: A Novel Approach to Improve Bioavailability

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Abstract

Background: Tuberculosis (TB), caused by Mycobacterium tuberculosis, is a highly infectious airborne disease affecting one-third of the global population and causing over one million deaths annually. While primarily targeting the lungs, TB can affect multiple organs, posing a significant global health burden. This study explores lipid drug conjugation as a novel targeted therapy for TB. Aerosolization or inhalation of lipid nanoparticle-based colloidal systems offers a promising approach for pulmonary drug delivery, directly targeting the lungs, where TB infection originates. Methods: A tripartite solid lipid nanoparticle (SLN)-based anti-TB formulation was developed using the solvent diffusion technique for prolonged lung delivery. This combination therapy incorporated Isoniazid (INH), Rifampicin (RIF), and Pyrazinamide (PYZ)—the gold-standard TB treatment regimen. Results: The formulated SLNs demonstrated an encapsulation efficiency of 40.53% to 63.73%. Physicochemical characterization, including particle size analysis and transmission electron microscopy (TEM), confirmed spherical nanoparticles with a smooth surface, measuring between 345.0 nm and 640.6 nm. The drug release profile was pH-dependent. Notably, SLNs exhibited exceptional long-term stability. In vivo studies revealed prolonged lung retention compared to nebulized nasal solutions, indicating effective pulmonary targeting and sustained drug release in the bronchiole tree. Conclusion: The developed lipoidal nanoparticles enhanced the biopharmaceutical properties of anti-TB drugs, offering a promising strategy for targeted pulmonary therapy.

Keywords: Solid lipid nanoparticles, Pulmonary drug delivery, Tuberculosis treatment, Rifampicin bioavailability, Inhalable nano-formulation

Significance | Solid lipid nanoparticles improved anti-tubercular drug encapsulation, stability, and lung retention, offering a promising targeted pulmonary delivery approach for tuberculosis treatment.

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Introduction

Tuberculosis (TB) is one of the oldest chronic recurrent infectious diseases caused by the airborne organism Mycobacterium tuberculosis (Bloom et al., 2017; Bomanji et al., 2015). TB, infecting approximately 1 in 3 people throughout the world, causes over 1 million deaths every year (Addio et al., 2015). The disease is spread in humans via the respiratory route and primarily attacks the lungs tissue (Bloom et al., 2017). It can affect several vital organs, including the lungs, brain, kidney, spleen, and bone marrow,

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resulting in a major dead load per year (Smith, 2003). An increase in multidrug-resistant TB among patients suffering from AIDS/HIV is emerging as a worsening scenario (World Health Organization [WHO], 2020).

Conventional treatment of pulmonary TB involves chronic oral administration of high-dose antimicrobials, which are associated with widespread adverse effects such as incompatibility, hepatotoxicity, and lack of patient compliance, leading to therapy failure and resurgence of the infection (Varghese et al., 2018; Nasiruddin et al., 2017; Ramappa & Aithal, 2013). The majority of drug molecules administered in conventional treatment do not reach their specific site of action and accumulate in the body for a long time (Varghese et al., 2018). Although many conventional formulations are available in the marketplace, they suffer from systemic toxicity upon prolonged use due to nonspecific biodistribution. WHO data show that the combined therapy of isoniazid (INH), rifampicin (RIF), and pyrazinamide (PYZ) remains the gold standard for treating TB, despite the introduction of new medications and anti-tubercular drug regimens (Blomberg et al., 2001).

Researchers have reported poly (dl-lactide-co-glycolide) nanoparticles for encapsulation of antitubercular drugs (RIF, INH, and PYZ) with the aim of reducing dosing frequency for better disease management (Pandey et al., 2003a; Pandey et al., 2003b). Zahoor et al. (2005) observed that when the same antitubercular drugs were encapsulated in alginate nanoparticles and administered via inhalation, they were more effective than free oral drugs in guinea pigs. Furthermore, in a mouse model, Pandey and Khuller (2006) investigated cerebral drug delivery of oral poly-lactide-co-glycolide nanoparticle-encapsulated antitubercular medicines, specifically RIF, INH, PYZ, and ethambutol (ETH). To administer anti-tubercular medications at a sustained pace, Maya et al. (2015) synthesized polymeric nanoparticles containing RIF, INH, PYZ, and ETH using a modified solvent emulsification technique. A series of qualitative in vivo investigations on alveolar macrophages revealed that nanoparticles were endocytosed with no significant adsorption. However, to determine the pharmacokinetic potential of the discovered polymeric nanoparticles, they suggested further research.

Hence, retention time in the lungs plays a key role in the development of lung-targeted nanoparticles. Regarding their size and retention ability (Zhao et al., 2017), inhaled solid lipid nanoparticles (SLNs) can extend drug release to maintain a constant concentration at the infection site, which would be impossible to achieve otherwise due to the high blood perfusion rate in the lungs. Few investigations have been reported to date on SLNs with antitubercular drugs (Pandey et al., 2005; Khatak et al., 2018). Most of these formulations are based on oral delivery and often suffer from first-pass metabolism and systemic toxicity (Pal et al., 2019; Gelperina et al., 2005). The potential benefits of site-specific delivery via inhalation of antitubercular drugs to the lungs include reduced systemic toxicity and higher drug concentration at the primary infection site. Additionally, a primary advantage of inhalation is that the drugs are not prone to first-pass metabolism (Gelperina et al., 2005).

However, a gap remains in the formulation strategies developed by previous researchers. Multiple drug treatments are restricted in many cases due to the degradation of RIF, leading to its decreased bioavailability (Singh et al., 2001), thereby necessitating an increased dose or the incorporation of antioxidants. Evidence suggests that RIF degrades in the gastric environment, a process exacerbated by the presence of INH (Shishoo et al., 1999), although PYZ remains stable. RIF hydrolyzes in acidic pH to produce a poorly absorbed 3-formylrifamycin (3-FRSV) moiety, which reacts with INH to form isonicotinyl hydrazone. This compound is then converted back to INH and 3-FRSV via first-order kinetics, leading to significant RIF loss and reduced bioavailability, while INH is recovered (Singh et al., 2001; Shishoo et al., 1999; Freire et al., 2014). Therefore, segregated delivery of RIF and INH for improved RIF bioavailability could be a step toward addressing treatment failure and increasing patient compliance. Additionally, studies have shown that T-lymphocytes are inhibited in the presence of RIF in both TB patients and healthy volunteers without significant differences (Gupta et al., 1975). Notably, a study by Tousif et al. (2014) found that INH successfully induced apoptosis of activated CD4+ T cells, increasing the chances of TB recurrence. Thus, reducing the dose of these drugs in combined TB chemotherapy could lead to more effective treatment while lowering the risk of reinfection.

In the present study, an effort was made to develop a nanoparticle-based anti-tubercular formulation for systemic delivery over a prolonged period, with special attention to lung-targeted delivery via inhalation. This study aimed to prepare SLNs of anti-tubercular agents like INH, RIF, and PYZ in separate lipid cores to retain the nanoparticles at the pulmonary infection site for a prolonged duration and provide a locally high drug concentration to combat disease progression. By extending the dosage interval or modifying the dose, this approach may improve drug utilization and reduce systemic toxicity. Physicochemical characterization of the prepared SLNs was performed using particle size analysis and transmission electron microscopy (TEM). Additionally, in vitro drug release studies, antimicrobial assays, cytotoxicity studies, in vivo lung deposition, and pharmacokinetic studies were conducted. The work has been patented under the Federal Republic of Germany: "Auf Lipid-Nanopartikeln basierende antituberkulose Arzneimittelzusammensetzung mit verbesserten biopharmazeutischen Eigenschaften (Lipid nanoparticle-based

anti-tuberculous drug composition with improved biopharmaceutical properties) IPC-A61K 9/14."

2. Materials and Methods

2.1. Materials

Isoniazid, Pyrazinamide, and Rifampicin were generously gifted by M/s Cadila Pharmaceuticals, Ahmedabad, India. Polyvinyl alcohol (PVA), Stearic acid, and Mannitol were procured from Sigma-Aldrich, USA. Tristearin, Soya lecithin, and Tween 80 were purchased from Himedia, India. Span 60 was purchased from Loba Chem., India. Compritol 888ATO was obtained from Gattifosse, Germany as a gift sample. The remaining reagents and chemicals were all analytical grade and were utilized as supplied, requiring no additional purification.

2.2. Development of Solid Lipid Nanoparticle (SLN) of Isoniazid (INH) and Pyrazinamide (PYZ)

Isoniazid and pyrazinamide SLN were produced using the previously described emulsion solvent diffusion process, with a few minor adjustments (Pandey, Sharma, & Khuller, 2005; Maya, Mishra, & Chawla, 2015). In brief, the lipid was heated above its melting point on a water bath. The drug and surfactant pre-mix in ethanol and chloroform (1:1) were added to the molten lipid phase and dissolved completely in a hot condition. The resultant organic phase was added to an aqueous polyvinyl alcohol (PVA) solution chilled to 4°C–8°C. The mixture was homogenized (Ultra-Turrax, IKA, Germany) for 15 minutes at 18,000 rpm after being mechanically stirred for 30 minutes. Subsequently, the organic solvents were eliminated using a probe sonicator for 15 minutes, which also allowed for additional particle size reduction and degassing. The resulting SLNs were retrieved by centrifugation using a high-speed cooling centrifuge (Remi 24 CRP PLUS, India) at 18,000 rpm for 30 minutes at 4°C. After three rounds of distilled water washing to remove any remaining PVA, the recovered SLNs were lyophilized (Shin PVTFD10R, IIC Industrial Corporation, India), with mannitol acting as a lyoprotectant. Table I outlines the formulation compositions.

2.3. Fabrication of Lipid Drug Complex of Rifampicin-loaded SLN

2.3.1. Development of Lipid Drug Complex of Rifampicin

The dissolving approach was used to prepare the lipid drug complex (LDC). The medication and lipid were dissolved in an organic or non-aqueous solvent. A light-protected environment was used to synthesize a 1:1 molar ratio of rifampicin and stearic acid, with ethanol serving as the solvent. The mixture was agitated in a rotamantle (Remi Motors, India) at no more than 75°C for one full day. The RIF-LDC was produced as a dry residue when the solvent had evaporated in a rotary evaporator (RV10; IKA, Germany) (Müller & Olbrich, 2004; Singh, Bhatt, Gill, & Suresh, 2014).

2.3.2. Fabrication of RIF-LDC Nanoparticle

The emulsion solvent diffusion method was utilized to generate lipid drug conjugate nanoparticles, which were further developed into solid lipid nanoparticles with minor modifications to previously published methodologies (Pandey, Sharma, & Khuller, 2005; Zhao et al., 2017). In summary, the RIF-LDC was precisely weighed (0.05 gm LDC equals 0.046 g RIF). After dissolving the lipid and RIF-LDC in an organic solvent, the mixture was heated to 60°–70°C in a water bath. Subsequently, the lipid phase was heated to the same temperature, and either or both emulsifiers—soy lecithin and span 60—were added. After adding the oil phase drop-by-drop to a 100 ml aqueous PVA solution, the mixture was homogenized for 15 minutes at 10,000 rpm using an Ultra-Turrax. The system was then placed in a bath sonicator for 45 minutes to break up any aggregates. The system was left overnight with magnetic stirring to ensure that all the organic solvent had been eliminated. After that, a cooling centrifuge was used to centrifuge the solid lipid nanoparticle dispersion at 18,000 rpm. After removing the pellets from the supernatant, 10% mannitol was added as a lyoprotectant, and the mixture was stored at -20°C in a petridish. The formulation was then lyophilized. Table 1 lists their compositions. Additionally, SLNs containing pure RIF were developed using the same procedure.

2.4. Evaluation of Developed Formulation

2.4.1. Particle Size and Zeta Potential

The average particle size, size distribution, polydispersity index (PDI), and zeta potential of the produced nanoparticles were ascertained using Zetasizer Nano-ZS90 (Malvern, UK) quasi-elastic laser light diffraction. Measurements were made by dissolving a weighed quantity of the experimental sample in distilled water and placing it in a specific cuvette (Pal, Adhikary, Ray, Das, & Mazumder, 2018).

2.4.2. Transmission Electron Microscopy (TEM) of SLN

To prepare the sample for TEM imaging, around 1 milligram of the lyophilized formulation was dissolved in 600 µl of Milli-Q water in an eppendorf tube, and one drop of the mixture was placed onto a copper grid. After allowing the material to air dry, it was examined using a transmission electron microscope (FEI, Tecnai G2 Spirit Bio TWIN, Czech Republic) operating at 50–60 Hz and voltage of approximately 210–240 V (Bhattacharjee et al., 2020; Das et al., 2015).

2.5. Drug Entrapment Efficiency

The drug entrapment efficiency was determined using the SLN powders. An Eppendorf tube filled with an appropriate organic solvent (methanol for R1 & P9, methanol-chloroform mixture for I9) was filled with an accurately weighted quantity of lyophilized SLNs, and the tube was forcefully vortexed for one hour. After filtering, the amount of drug in the filtrate was measured at each drug's λmax using a UV-Vis spectrophotometer (UV-1800,

Shimadzu, Japan). The effectiveness of drug encapsulation was ascertained in this way.

2.6. In vitro Drug Release and Release Kinetics Study

An in vitro drug release study was conducted for 12 hours in order to examine the drug release at different times. In order to replicate various interstitial conditions in the healthy lung, artificial lysosomal fluid (ALF) pH 4.5 and Gamble's solution pH 7.4 have been employed as dissolving media, while 0.1(N) HCl has been used to represent the sick state of the lungs, as any disease condition induces a more acidic pH in the adjacent environment. ALF and Gamble's fluid were prepared by the method described by Marques et al. (2011). This was accomplished by suspending a weighed quantity of the produced nanoparticles (I9, R1, and P9) in 100 milliliters of the corresponding fluid and stirring them magnetically while keeping the temperature at approximately 37°C ± 1°C. Samples were removed, filtered through a 0.45 µm membrane filter, and diluted with fresh medium as needed at prearranged intervals of 15 minutes, 30 minutes, 1, 2, 3, 4, 5, 6, 8, 10, and 12 hours. To keep the sink in working order, a fresh medium of the same volume was added. UV-Visible spectroscopy (UV-1800, Shimadzu, Japan) was used to measure the amount of drug released according to each medication's λmax. The total percentage of drug release against time was plotted for the drug released from the formulations. To clarify the process of drug release from the nanoparticles, data from investigations on in vitro release were fitted to a variety of kinetic models.

2.7. Antimicrobial Assay

Mycobacterium tuberculosis H37Rv (ATCC 27294) cultures were maintained on Middlebrook 7H11 Media slant at 37°C. After 21 days of incubation, bacterial cells were scraped from slants and diluted to obtain a growth index of approximately 1×106 cfu/mL. This was used as inoculum. Each sample was taken on a weight basis and diluted using DMSO to obtain concentrations of 500, 250, 100, 50, and 25 µg/mL. Agar diffusion studies were conducted using sterilized petri plates with standard drugs. The samples were tested for their antitubercular activity individually. The plates were kept for diffusion and placed in a negative air pressure chamber, in a 5-10% CO2 atmosphere at 37°C for 4 days, and the zone of inhibition was measured. All sets of experiments were conducted in triplicate. The same method was modified in terms of incubation time extension if suitable results were not achieved. Separate dilutions were made for MIC measurement, where necessary and calculated thereafter (Petersdorf & Sherris, 1965).

2.8. Cytotoxicity Assay

The cytotoxicity of the optimized formulation was evaluated by an in vitro assay as described by Mosmann (1983) with slight modifications. L6 (rat skeletal myoblast) and A549 (adenocarcinomic human alveolar basal epithelial) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) and Minimum Essential Medium Eagle (MEM) medium, respectively. These media were supplemented with 10% fetal bovine serum (FBS), 10% Penstrep, and 1% gentamicin. In summary, complete media was used to seed 1 × 106 cells per milliliter in tissue culture grade multiwell plates, and standard procedures were followed for incubation at 37°C in a humidified 5% CO2 atmosphere. Once a full day had passed, the medium was switched out for FBS-free medium and incubated for another night. Following that, the cells were treated with the optimized RIF-LDC at various concentrations in each well and incubated for 24 and 48 hours, respectively. The control was the well-contained media (untreated cells). Following the incubation period, 10 µL of MTT (5 mg/mL) was transferred to each well, gently mixed, and then allowed to incubate for an additional 4 hours. After the period of incubation, the cells were examined using an inverted microscope to check for the existence of formazan crystals that were dark purple and located at the well's bottom. Each well received 100 µL of isopropanol and 0.04 N HCl, which were pipetted in repeatedly using a multichannel pipette to ensure thorough mixing. The phenol red in the tissue culture media is changed to a yellow hue by the HCl, which has no effect on the MTT formazan assay. The formazan is dissolved by the isopropanol, producing a uniform blue solution that may be used to measure absorbance. An ELISA plate reader (FilterMax F3 Multi-Mode Microplate Readers, Molecular Devices) was used to measure the absorbance. The test wavelength was 570 nm, while the reference wavelength was 630 nm. Every experiment was run in triplicate. The impact of the optimized RIF-LDC on cell proliferation was quantified as the percentage of viable cells, which was computed as follows:

(Absorbance of treated cells / Absorbance of control cells) × 100.

2.9. In-vivo Lung Deposition Study and Pharmacokinetic Study

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC approval no: IAEC/DU/54 dated 24.09.2013). The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) declared the principles that the animals were kept in line with; Regd No. 1576/go/a/11/CPCSEA dated 17.02.2012. The animals were housed at the Central Animal House Facility of Dibrugarh University, maintained under the supervision of the Department of Pharmaceutical Sciences. Swiss albino mice, aged 4-6 weeks and weighing 15-20 gm, of both sexes, were used for the study. The animals were kept in standard cages with laboratory conditions such as a temperature of 19–25°C, relative humidity 50–70%, and a 12-hour light and dark cycle. They were supplied with an appropriate diet and water ad libitum throughout the study period. SLN containing INH was administered at a dose of 10 mg/kg body weight, PYZ at 25 mg/kg body weight, and LDC-RIF at 12 mg/kg body weight of animals (Pandey et al., 2003a, 2003b).

Before exposing the experimental animals to the formulations, SLNs were mixed with microfine lactose in a 1:3 ratio and mixed well to obtain a free-flowing powder suitable for inhalation delivery. The final experimental formulations were administered to the animals by inhalation using a nose-only chamber, fabricated in-house following the model proposed by Sinha and Mukherjee (2012). For the lung deposition study, animals were divided into three groups for each drug, each containing four animals. After exposure to the formulations by inhalation, three animals from each group were sacrificed at specific time points, and their lungs were extracted. To assess the drug concentration in the pulmonary tissue (Das et al., 2015), the lungs were homogenized with saline, and the drug was extracted using a suitable solvent after protein precipitation. The samples were dried under vacuum and analyzed by HPLC.

For the pharmacokinetic study (Pandey et al., 2003b), the experimental animals were divided into two groups, each containing six animals. These animals were exposed to lyophilized drug solutions and dry optimized formulations via inhalation. Blood samples were collected by retro-orbital sinus at specific time intervals. The samples were centrifuged at 4°C under 5000 rpm to separate the plasma. The drugs were extracted with a suitable organic solvent, and the samples were dried under vacuum. Afterward, the dried samples were diluted with mobile phases and analyzed by HPLC. Pharmacokinetic parameters, including plasma concentration, Tmax, Cmax, AUC, and biological t1/2, were determined.

3. Results and Discussions

3.1. Development of SLN Formulation

All SLN formulations were prepared successfully using the solvent emulsion evaporation technique. Isoniazid (INH) is an active pharmaceutical ingredient (API) that is positioned between BCS Class I and III due to its low permeability (Becker et al., 2007). To address this issue, an SLN formulation was developed with a lipid matrix consisting of tristearin and Compritol 888 ATO. The inclusion of Compritol 888 ATO alongside tristearin helped achieve an adequate drug loading in the formulation. It was found that changes in the concentration of Tween 80 did not affect the drug entrapment efficiency of the formulations, so it was maintained at a constant concentration. An increase in PVA concentration resulted in a higher percentage yield of the formulations, along with an improvement in drug entrapment efficiency.

When SLNs were developed with pure rifampicin (RIF), a BCS Class II API (Becker et al., 2009), the drug entrapment efficiency (DEE) was found to be low (15.38%) due to the poor solubility of the molecule (Agrawal & Panchagnula, 2005). To enhance the entrapment of RIF, a Rifampicin-Lipid Drug Complex (RIF-LDC) was developed (Singh et al., 2014). Rifampicin and stearic acid reacted in a 1:1 molar ratio to form a drug-lipid complex, which was then incorporated into the SLN formulation. Tristearin was used as the lipid matrix, while a combination of Span 60 and soya lecithin helped develop a stable formulation, with PVA acting as a stabilizer. The increase in total surfactant concentration was shown to improve both the percentage yield and drug entrapment efficiency, meeting the primary goal of the formulation development study.

For pyrazinamide (PYZ), a distinctly BCS Class III drug facing low permeability (Becker et al., 2008), the need for a targeted delivery system was evident. In this study, PYZ SLNs were formulated using Compritol 888 ATO as the lipid matrix, a combination of Tween 80 and Span 60 as surfactants, and aqueous PVA as the stabilizer. It was observed that changes in Tween 80 concentration did not significantly affect the entrapment of PYZ in the SLNs. However, increasing the concentration of Span 60 led to an improvement in the entrapment efficiency of PYZ in the SLN formulation.

3.2. Characterization of Solid Lipid Nanoparticles

3.2.1. Particle Size and Zeta Potential

The zeta potential, polydispersity index (PDI), and particle size of each SLN formulation (I9, R1, and P9) were assessed. Colloidal formulations are considered stable when their zeta potential is greater than 30 mV or close to -30 mV. The lower the PDI, the more monodispersed the formulation. The optimized formulations were found to be stable upon lyophilization and were monodispersed, with particle sizes ranging from 300 nm to 600 nm, as indicated by the PDI values (data not included in Table I). The biological distribution of SLNs throughout the body is significantly influenced by particle size (Mukherjee, Ray, & Thakur, 2009). The zeta potential of SLNs significantly affects their pharmacokinetic and biodistribution patterns. Positively charged particles are filtered out of the body more rapidly at the glomerulus' basement membrane than negatively charged ones (Haraldsson et al., 2008). All the SLNs had negative zeta potential values, ranging from -4.62 to -26.4, which suggests that electrostatic repulsion contributes to the stability of the nanoparticles (Gaur et al., 2014).

3.2.2. TEM of SLNs

Transmission Electron Microscopy (TEM) is used to examine the surface morphology of particles. TEM images (Figure. I) of the SLNs revealed that all particles were nanometric in size, consistent with the results of particle size distribution. The SLNs were found to have smooth, even surfaces and spherical shapes. Additionally, no aggregation was observed, which correlates well with the negative ζ-potential values (Maji et al., 2014).

3.3. Drug Release Study and Release Kinetics

Artificial Lung Fluid (ALF, pH 4.5) was used for the dissolution study, as it simulates the fluid that nanoparticles encounter after being phagocytosed by lung interstitial and alveolar macrophages. Gamble's solution, with a pH of 7.4, mimics the lung's interstitial fluid in a healthy state. These pH values are comparable to normal

lungs, while the bronchus in diseased conditions presents a higher pH (Bhattacharjee et al., 2020). For the dissolution investigation, nanoparticles were exposed to 0.1(N) HCl to simulate a diseased lung condition. SLN and LDC nanoparticles with the highest DEE were subjected to the drug release study.

Rifampicin release from RIF-LDC SLN is expected to occur in two stages: first, the SLN releases the LDC, and then the LDC releases rifampicin (Müller & Olbrich, 2004). As RIF-LDC serves as a prodrug, it is necessary for the LDC to release RIF at the drug delivery site. The release of RIF from RIF-LDC and pure drug was studied in simulated lung fluid (SLF), and the results showed that LDC delayed RIF release compared to its pure drug. After 150 minutes, 78% of RIF was released from LDC.

In a 12-hour in vitro dissolution study in Gamble's solution, formulation I9 released 82.3% isoniazid, formulation R1 released 84.54% rifampicin, and formulation P9 released 78.54% pyrazinamide. All three formulations showed a pH-dependent release, with faster release observed at more acidic pH. The release of drug from all three formulations in vitro, when exposed to 0.1(N) HCl, was faster. I9 released 100% isoniazid in 11 hours, while R1 and P9 released 98.99% rifampicin and pyrazinamide, respectively, within 10 hours, with complete drug release observed by the 11th hour. It can be concluded that in a diseased lung environment, the nanoparticles will fully release their drug payloads, aiding in achieving the minimum inhibitory concentration (MIC).

The drug release mechanism from nanoparticles primarily followed the Korsmeyer-Pappas model, with drug release kinetics in SLF (pH 7.4), ALF (pH 4.5), and 0.1(N) HCl following the Higuchi and zero-order models. Some cases showed first-order kinetics, indicating that the drug release from the SLNs was controlled by a combination of diffusion and erosion processes, or non-Fickian diffusion, depending on the value of "n" (Pal et al., 2018).

3.4. Drug Entrapment Efficiency

Table I demonstrates that formulation I9 achieved the maximum drug entrapment efficiency (DEE), i.e., 56.34% for isoniazid (INH), 63.73% for rifampicin (RIF), and 57.78% for pyrazinamide (PYZ). It was observed that, for INH solid lipid nanoparticles (SLNs), increasing the PVA concentration from 0.25% to 1% aqueous solution aided in achieving higher drug entrapment efficiency, from 42.59% to 56.34%, while keeping lipid and surfactant concentrations constant. In contrast, in the case of PYZ SLNs, changes in PVA concentration did not affect the DEE, so these data are not shown. However, a gradual increase in span 60 concentration led to a higher DEE, from 40.53% to 57.78%, without changing the lipid concentration in the formulation. In the case of RIF, simple RIF-loaded SLNs exhibited very low drug entrapment compared to RIF-LDC-SLNs. The DEE increased with variation in s in SLNs, which was further enhanced with an increase in the total surfactant concentration in the formulation.

Overall, it was observed that an increase in drug entrapment was achieved with the increasing concentration of either surfactant or PVA. This can be attributed to various theories of drug solubilization. Drugs with intermediate solubility, like RIF, can be better entrapped in the palisade layer of the surfactants (Rangel-Yagui et al., 2005; Kim et al., 2001). On the other hand, INH and PYZ, being polar in nature, are likely to be attached rather than entrapped in the hydrophilic heads of surfactants (Kim et al., 2001; Rangel-Yagui et al., 2005).

3.5. Antimicrobial Assay of the Optimized SLNs

The formulations were tested for their antitubercular activity individually in triplicate. The minimum inhibitory concentration (MIC) was calculated from the zone of inhibition and is reported in Table II. The results show that the drug loading in the formulations was adequate to exhibit anti-TB activity against the Mycobacterium tuberculosis H37Rv strain. For example, a 500 µg/mL INH SLN actually contains 141 µg/mL of drug. The detailed MIC values for each formulation are provided in Table II.

3.6. Cytotoxicity Assay of the Optimized SLNs

In today's era, evaluating the cytotoxicity of novel formulations is an obligatory step to assess their safety before commencement of in vivo toxicity studies (Gaspar et al., 2016). While a precise determination of toxicity can only be achieved through in vivo acute toxicity studies, in vitro cytotoxicity assays are widely accepted as preliminary indicators of safety. For this purpose, the optimized SLNs were evaluated for cytotoxicity using the MTT assay, which measures the metabolic activity of cells. The SLNs were prepared from FDA-approved biocompatible and biodegradable lipids with Generally Recognized as Safe (GRAS) status, offering better tolerability compared to polymeric or metallic nanoparticles. These lipids also serve as a nutritional source when the drug is released at the target site (Adhikari et al., 2017).

To investigate potential cytotoxicity, the optimized formulations (INH-SLN, RIF-LDC-SLN, and PYZ-SLN) were exposed to L6 (rat skeletal myoblast) and A549 (adenocarcinomic human alveolar basal epithelial) cell lines for 24 and 48 hours. A549 cells are representative of the alveolar epithelium, while L6 cells serve as a model for healthy cells (Rodrigues et al., 2015). The MTT assay assesses cell viability by evaluating the reduction of MTT reagent to tetrazolium salts, a process dependent on mitochondrial metabolism (Gaspar et al., 2016). In this study, the cell viability was observed to remain greater than 85% when exposed to a concentration of 50 µg/mL for both cell lines, and 50% cell viability was observed even at a concentration as high as 600 µg/mL. As expected, higher concentrations and extended incubation times led

Table 1. Composition and Characterization of SLN formulations.

Code	Drug	Lipid/Surfactant	Lipid /Drug Ratio (w/w)	Lipid/ Surfactant Ratio (w/w)	PVA %	% Yield	Z-ave (nm)	ZP (mV)	EE (%)

Table IV. Pharmacokinetic parameter

PARAMETERS	ISONIAZID		PYRAZINAMIDE		RIFAMPICIN	
	SOL	SLN	SOL	SLN	SOL	SLN
$t_{1/2}$(d)	1.26 ±0.952	2.46 ±0.52*	1.59 ±	1.840±0.992	1.602±0.472	1.955±0.893*

to decreased cell viability, but the viability remained above 50%. The LD50 values, which are an indicator of cytotoxicity (with

higher LD50 values indicating lower cytotoxicity), were calculated and are reported in Table III. The high LD50 values and cell viability suggest that the optimized formulations do not pose significant cytotoxicity, indicating their safety for further testing.

3.7. In-vivo Lung Deposition Study and Pharmacokinetic study

In vivo lung deposition study showed that adequate deposition of drugs, i.e., isoniazid, rifampicin and pyrazinamide was achieved from the formulations. From Figure. III, it can be observed that for all the three drugs, maximum lung concentration was found in 3rd h after dosing (1.902 g/ml for INH, 2.825 g/ml for RIF and 2.895 g/ml for PYZ), which eventually reduced at 6th h followed by 12th h gradually. Similar drug clearance profile from lungs was observed in a study conducted by Pandey and Khuller (2006) . From this pattern we can infer that, the drugs are slowly entering systemic circulation after leaving the alveolar net like structure of lungs. Presence of drugs in the lungs up to 12 hours proved the prolonged retention of nanoparticles at the infection site, which was the main objective of the present study. This will help to provide high drug concentration to combat the detrimental progress of the disease. Because of its size, SLN will boost the concentration of the drugs at the infection site, and its prolonged release will aid to maintain the steady state concentration in the lungs, something that other drug delivery methods cannot accomplish because of the high blood perfusion rate in the lungs. By extending the interval between doses or changing the dosage, this will also lessen systemic toxicity and promote improved use of the drug.

Pharmacokinetic study was carried on two groups of animals, where one group received drug solution and the other group received test formulation, both being administered by inhalation route. The plasma concentration-time profiles of each drug have been shown in Figure. IV (a, b, c) for Isoniazid, Rifampicin and Pyrazinamide. The pharmacokinetic parameters that were evaluated namely, t1/2, Tmax, Cmax, AUC0-t, AUC0-inf_obs, AUMC0-inf_obs, MRT0-inf_obs, Cl_obs and Vss_obs (Vd) have been tabulated in Table 4 with their values. It is also evident that SLN prolonged the drug release and lung retention than drug solution as Cmax was reached in 2 days in case of all SLN formulations, but the same was reached in 1 day in case of each drug solutions, although Cmax remained almost same. The SLN being a lipid-based formulation, it is likely that lipophilic drugs would remain incorporated for a longer time period whereas hydrophilic drugs would be released more. Henceforward, Rifampicin (a hydrophobic drug) was released to a lesser extent in serum as compared with the hydrophilic drugs, isoniazid/pyrazinamide. Isoniazid and pyrazinamide medications were found in the plasma of rats that had received a single inhalation of ATD-loaded SLNs starting at day 0.5 and continuing until day 6 (Figure.IV). Rifampicin, however, was discovered 0.25 days later. In contrast, free drugs (SOL) were detectable in plasma as early as 0.12 days after inhalational delivery; however, they were not detectable in plasma beyond day 5. Once the SLNs were inhaled, they were deposited in the lungs, where the medicines were released and circulated to the different extrapulmonary organs. Mucociliary clearance of the particles in the lungs was also seen. This meant that as time went on, it was anticipated that medication levels in the liver and spleen would rise while those in the lungs would fall. Because of the slow and sustained release of drugs from the SLNs, the MRT was found to be increased significantly by 2-3-fold (***p<0.001) compared to the inhaled free drug (SOL) (Table x). In addition, Tmax (d) was also found to be doubled for SLN as compared to SOL (*p<0.05) further confirming the sustained release of all the ATDs. This resulted in an improved AUC 0-inf_obs of all the drugs as has been established in this study. Furthermore, inhalation is recognized to progress drug pharmacokinetic parameters which is also supported by our results (Table IV). However, there were no significant changes observed for Cmax (µg/ml) and clearance (Cl_obs) of the drugs. In a nutshell, there was observed a significantly better pharmacokinetic profile of the ATDs when fabricated as SLNs compared to SOL in this present study. Considering that all three medications were still present in the blood plasma six days after inhalation (Figure. IV), an antitubercular chemotherapy schedule could be created that would substitute weekly inhalation of drug-loaded solid lung nanoparticles for daily oral administration (conventional therapy) over a period of six weeks.

3.8. Stability study of developed SLN:

The long-term stability studies of the prepared lipid drug complex was performed as per the draft ICH Guidelines for products to be stored under refrigeration. The three formulations of Isoniazid(I9), Rifampicin(R1) and pyrazinamide(P9) were packed in separate vials and stored in at 5 o C (Under refrigeration) for a period of 12 months. Any change in average particle size upon storage with time, and modification in physical appearance of developed SLNs are reasonable sign of changes in kinetic stability. The formulations were checked at an interval of 3,6, 9 and 12 months and change in physical appearance of the SLNs were examined. All the three formulations I9, R1, P9, were absolutely stable throughout the duration of storage period of 12 months and all the critical parameters like zeta potential, particle size, PDI and drug release were within permitted range and there was not much difference in comparison to the parameters evaluated at month 0. The study results indicate that the formulation has substantial stability indicating a good shelf life and can be scaled up to large scale formulation batches. Moreover, for large scale manufacturing the length of anticipated storage period (expiration date) under these conditions will depend on related factors. The present formulation showed a remarkable stability profile for the said study period of twelve months. The stability study conditions is mentioned in data

is presented in Table V and the results of stability study is presented in Table VI.

4. Conclusion

As reviewed by Gordillo-Galeano A. et al [54] SLNs have wide potential in lungs targeted antitubercular treatment by altering the biopharmaceutical attributes of the dose regimen. In the present study, three nano lipid formulations containing Isoniazid, Rifampicin and Pyrazinamide were successfully prepared, and their combination dose which is suitable for inhalation was also developed by mixing with micro fine lactose for lung targeting. The ideal particle size of the delivery system for lung targeting should be greater than 400 nm and less than 2.5 m in order to be kept in the alveolar sac. It is anticipated that the aerodynamic diameter of the nanoparticles will grow following processing of the nano-formulations using lactose along with additional excipients to make it a suitable DPI for energetic lung targeting. The developed lung targeted SLNs bear the advantage of avoiding the fast pass metabolism as it avoids oral route. Further, the spherical smooth and even surface morphology of optimized formulations were found to be satisfactory and drug entrapment efficiency saw high enough to maintain the minimum effective concentrations of the drugs at the site of infection. The cytotoxicity study of the formulations proved that the developed nanoparticles were safe and non-toxic with high LD50 value. In vivo studies reviled that adequate lung retention of all the drugs was achieved and drugs could be retained in lungs for a longer time when optimized SLN formulations were administered than pure drug solution by inhalation route. Hence, it can be concluded that the aim of developing a lipoidal nanoparticulate system of three antitubercular drugs to address their biopharmaceutical challenges was successfully achieved.

Author contributions

P. P. contributed to the conceptualization, methodology, investigation, study design, data revising, and writing of the original draft. S. R. was involved in the conceptualization and study design. A.K. D. was responsible for analysis and data interpretation, writing the original draft, as well as review, editing, and visualization. M.S.K. and S. D. contributed to the investigation, formal analysis, and validation of the study. P.P.D. collaborated on the investigation and study design. T. S. and M. S. contributed to the review, editing, and visualization. Finally, B. M. supervised the project, contributed to the conceptualization and study design, and managed the project administration.

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Compliance with Ethical standards

All institutional guidelines for care and use of laboratory animals were followed in this research.

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Competing financial interests

The authors have no conflict of interest.

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