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## Investigation of Antimycobacterial potential of solid lipid nanoparticles against *M. smegmatis*

**Sunil Khatak, Uttam Prasad Semwal, Manoj Kumar Pandey and Harish Dureja**

### Abstract

The patient non-compliance and emergence of multidrug resistance (MDR) have great influence on the increased incidence of tuberculosis (TB). Nanotechnology-based chemotherapy improves pharmacokinetic behaviour, antimycobacterial efficacy reduces dosing frequency and cost of therapy. The present study, a modified microemulsion technique was utilized for the preparation of first-line antitubercular drugs (ATDs). i.e. rifampicin (RIF), isoniazid (INH) and pyrazinamide (PYZ) loaded solid lipid nanoparticles (SLNs). The morphology, particle size, zeta potential was characterized and antimycobacterial activity was evaluated against *Mycobacterium smegmatis* mc<sup>2</sup> 155 (*M. smegmatis*). The resulting ATDs loaded SLNs were spherical with average particle size 195.1±11.11 nm with negative zeta potential -41.9mV and 0.229 PDI. The percentage encapsulation efficiency of RIF, INH and PYZ were 84.6±2.11, 83.3±3.75 and 76.4±3.83, and loading capacity was found to be 43.8%, 35.4% and 39.7%, respectively. The practical yield was found to be 92.6%. The ATDs loaded SLNs formulation also showed sustained drug release for 24 h. The antimycobacterial activity was greatly enhanced against *M. smegmatis*, and the minimum inhibitory concentration (MIC) of ATDs loaded SLNs was approximately ten-times more effective as compared to standard drugs and two-times efficacy improvement as compared to a physical mixture of ATDs solution. Drug-free SLNs showed no antimycobacterial effect. It can be concluded that the SLNs are promising vehicles for the antimycobacterial activity of ATDs.

**Keywords:** Tuberculosis, Solid lipid nanoparticles, Rifampicin, Isoniazid, Pyrazinamide and *Mycobacterium smegmatis*

### 1. Introduction

Tuberculosis (TB) is a leading chronic disease infected by *M. tuberculosis* [1]. Tuberculosis is still a major health problem in many developing countries worldwide because of drug-resistant TB, as its treatment is longer and requires more expensive drugs. India is the second most populous country in the world and one-fourth of global incidence TB cases were found annually. WHO estimated the global annual incidence of 8.6 million TB cases and approx 2.3 million were estimated in India [2].

The conventional chemotherapy has various barriers such as poor bio-distribution at the cellular level, chemotherapy-associated side effects, drug-resistant tuberculosis and patient non-compliance (because of posology) results in treatment failure.

In the last decades, research groups worked to develop nanoparticle-based drug delivery systems for better management of antitubercular chemotherapy. This is because of unique physicochemical properties including the nano-size range, larger surface, high reactivity and unique interactions with the biological system. The various advantages of nanotechnology-based drug delivery science include enhanced drug serum solubility, prolonged systemic circulation lifetime, a sustained and controlled drug release, targeting drug delivery to the tissues and cells and concurrently multiple drugs therapeutic to the same cells [3-5]. Drug-loaded nanoparticles can enter into the host cell via endocytosis and then release drug payload to treat microbes-induced intracellular infections. As a result, a number of nanoparticles based drug delivery have been approved for clinical tests [3]. The nanoparticulate system has distinctive and relatively more effective drug delivery carriers, including liposomal-mediated drug delivery, polymeric nanoparticles/microparticles, solid lipid nanoparticles, nanosuspensions, nanoemulsions, niosomes, dendrimers, cyclodextrin inclusion complexes and other nanosystems exploiting the extraordinary properties of matter at the nanoscale. Nanoparticles have shown significant improvements in diagnosis, prevention,

treatment and provide the flexibility of selecting the invasive and non-invasive route of delivery of chemotherapy of tuberculosis. The researchers have much interest in nano-drug carriers as higher stability and great improvement of bioavailability and pharmacokinetics which further leads to a reduction in dosing frequency.

Solid lipid nanoparticles (SLNs) are colloidal particles of lipid matrix and are solid at both room and body temperature [6, 7]. SLNs have been investigated for various pharmaceutical applications for invasive and noninvasive drug delivery. SLNs loaded lipophilic and hydrophilic/amphiphilic drug formulae were utilized for drug targeting and controlled drug release [8]. SLNs can be prepared by using lipid(s), waxes and biocompatible surfactant(s) using various methods reported in the literature [9].

In the last two decades, SLNs have been reported as a potential carrier for chemotherapies and diagnostic applications because SLNs have several advantages over other colloidal carriers. The second generation lipid nanoparticles were also reported, they are made by modification in the composition of solid lipid and liquid lipid to overcome the limitation associated with SLNs. These second-generation lipid nanoparticles are Nanostructured Lipid Carriers (NLCs) and Lipid Drug Conjugates (LDCs) [9]. Chemotherapy of tuberculosis with first-line antitubercular drugs (ATDs) loaded SLNs improve patient compliance, this is a due to ultra size range, larger surface area, high reactivity and unique interactions with the biological system. The inhalation and oral administration of first-line ATDs loaded SLNs to mice and guinea pig model was showed great improvement in bioavailability and pharmacokinetic with reducing dosing frequency and improvement in the parameters [10–12]. The RIF loaded SLNs was showed significant improvement in antimycobacterial activity against *Mycobacterium fortuitum* (ATCC 2701P) and also sustained the drug release for 72 h [13]. Other studies were showed the improvement in the loading of INH in SLNs and prolonged drug release for a better therapeutic effect [14, 15].

In this study, the *M. smegmatis* mc<sup>2</sup> 155 has explored due to non-pathogenic, fast-growing *mycobacterium* and could serve as a surrogate for *M. tuberculosis*. In this pursuit, we have compared the antimycobacterial activity of standard drug solutions, a physical mixture of ATDs solution and ATDs loaded SLNs.

## 2. Materials and Methods

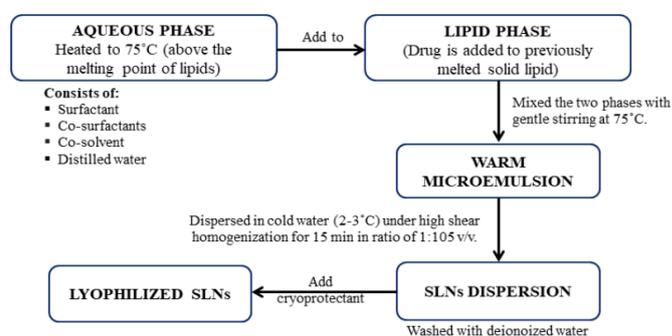
### 2.1 Materials

Rifampicin (RIF), Isoniazid (INH) and Pyrazinamide (PYZ) were obtained as gift samples from Indian Pharmacopoeia Commission (IPC), Ghaziabad, Uttar Pradesh, India. Soybean-Casein digests agar (SCDA) media for antimicrobial activity was purchased from Hi-media laboratories. *M. smegmatis* mc<sup>2</sup> 155 (mycobacterial stain) was collected as a gift sample from Dr. B.R. Ambedkar Center for Biomedical Research (ACBR), University of Delhi. Poloxamer 188 (Pluronic®F-68) was purchased from Hi-Media Laboratory Pvt. Ltd. India. Sodium taurocholate was purchased from LOBA Chemie, India. Stearic acid, Mannitol was purchased from Qualigens Fine Chemicals (Division of GSK Pharmaceutical Ltd.). Comperitol®888 ATO was obtained as gift sample from Gattefosse, France, and all other chemicals were of analytical reagent grade.

### 2.2 Preparation of SLNs

A modified microemulsion technique was utilized for the

preparation of ATDs loaded SLNs in this technique warm o/w microemulsion was dispersed in an ice-cold aqueous medium (Fig 1). The o/w microemulsion was prepared by dispersing the hot aqueous phase into the melted lipid phase. The phase consisted of poloxamer 188 (surfactant), sodium taurocholate (co-surfactant), ethanol (co-solvent) and deionized water added to 100% (w/w) and warmed upto 75°C. The oil phase, consisting of stearic acid and Compritol®888 ATO was heated to ~75°C. These two phases were mixed together at the temperature of 75°C with gentle stirring. The temperature was maintained above the melting point of stearic acid (58.97°C) and Compritol®888 ATO (73.42°C). The resultant clear microemulsion was immediately dispersed in an ice-cold water under high shear homogenization (IKA T-25 Ultra-Turrax digital high-speed homogenizer system) at maximum speed (8000 rpm) for 15 min in a ratio of 1:15 (v/v), respectively. Then, the SLNs dispersion was washed twice with deionized water using an ultracentrifuge (Sigma Aldrich, Switzerland). Finally, mannitol (Cryoprotectant) was added to the SLNs dispersion before lyophilization [16, 17].



**Fig 1:** Schematic representation of the microemulsion technique [18].

### 2.3 Incorporation of ATDs in SLNs

For incorporation of ATDs in SLNs, the lipids were heated around 75°C above its melting point and dissolved INH (100 mg), PYZ (250 mg) and RIF (120 mg) in molten lipids. Then a warm aqueous solution consisting of a surfactant, co-surfactant and co-solvent was added to obtain an optically transparent system. The temperature of the aqueous phase was also maintained at 75°C. This hot microemulsion was instantly disseminated in cold water (2–3°C) at 1:15 ratio of microemulsion/water (v/v) under high shear homogenization. The excess surfactant/co-surfactant was removed from SLNs dispersion by washing at least two times with deionized water. The mannitol a cryoprotectant was used before lyophilization to get dry powder of ATDs loaded SLNs for quantitative estimation.

### 2.4 Lyophilization

The 20 ml of SLNs dispersion was placed in a 40 ml wide mouth fast-freeze flask tube (FB 0040, IIShin) and placed the flask tube in ultra-low temperature freezer (DW-86L828J, ULT freezer, Haier) at –80°C. The frozen SLNs dispersion was lyophilized using a freeze dryer (IIShin Bio-base, Europe) at –80°C temperature and 20 mTorr pressure for 24 h.

### 2.5 Characterization of ATDs loaded SLNs

#### 2.5.1 Particle Size, PDI, zeta potential and morphology determinations

The mean particle size, polydispersity index (PDI) and zeta potential were determined at 25°C by using photon correlation

spectroscopy (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). The samples were diluted with deionized water before measurement to prevent interparticle scattering. The morphology of the SLNs was examined by JSM-6610LV scanning electron microscope (Jeol, USA). The nanoparticle sample was sprinkled on an aluminium stub having double adhesive carbon tape. The nanoparticles were then coated with gold to a thickness of 200 to 500 Å under an argon atmosphere with high vacuum evaporator. The nanoparticles were scanned at different magnifications and photomicrographs were taken out.

### 2.5.2 Entrapment efficiency, loading capacity and percentage yield measurement

Take 25 mg of ATDs loaded SLNs in 25 ml amber coloured volumetric flask and add 10 ml dimethyl sulfoxide (DMSO) to dissolve the contents with sonication for 30 min, and make up the volume upto 25 ml with DMSO followed by stirring for 10 min. After suitable dilution with buffer pH 6.8, the aliquot was subjected to HPLC analysis. The total drug content of ATDs loaded SLNs formulations was calculated by area under the curve (AUC) of the peak. The encapsulation efficiency, loading capacity and percentage yield were calculated by the following equations.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100$$

$$\text{Percentage yield} = \frac{\text{Total nanoparticle weight}}{\text{Total solid weight}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{Drug weight in the nanoparticles}}{\text{Total weight of nanoparticles}} \times 100$$

## 2.6 Antimycobacterial activity study

### 2.6.1 Preparation and dilution of standards

Prepare a standard stock solution of RIF, INH and PYZ by taking 7.78, 6.4 and 14.6 mg in 10 ml of dimethyl sulfoxide (DMSO) separately in 10 ml volumetric flasks. The stock solutions were serially diluted to estimate the minimum inhibitory concentration (MIC).

### 2.6.2 Preparation and dilution of the physical mixture of ATDs and ATDs loaded SLNs

To prepare the physical mixture of ATDs, take the equivalent amount (% EE of RIF, INH and PYZ in SLNs were 84.6, 83.3 and 76.4, respectively) that is 0.777, 0.64 and 1.46 mg of RIF, INH and PYZ respectively, dissolved in 5 ml of DMSO (considered as 200%). This solution was further diluted to prepare 100%, 75%, 50%, 25%, 12.5% and 6.25% solutions to estimate MIC. The procedure given above was utilized unchanged to prepare the dilutions of the ATDs loaded SLNs.

### 2.6.3 Diffusion method

The cup-plate method was employed to investigate the antimycobacterial activity of ATDs loaded SLNs. The dimethyl sulfoxide (DMSO) solution of working standards of ATDs, the physical mixture of ATDs (made exactly the same as ATDs loaded SLNs) and drug-free SLN (made exactly the same as ATDs loaded SLNs without adding the drugs) were used as control groups. *M. smegmatis* mc<sup>2</sup> 155 is a species in phylum *Actinobacteria* and the genus *Mycobacterium* was used for laboratory investigation due to its being a "fast-grower" and non-pathogenic. The transmittance of bacterial suspension was measured by UV spectrophotometer at 530

nm. The bacterial suspension (1.5%) with 25% transmittance was poured into the sterilized soybean casein digest agar (SCDA) medium at 40–45°C and mixed to homogenize<sup>[13]</sup>. The DMSO stock solution of ATDs (reference standards), physical mixture ATDs, freeze-dried ATDs loaded SLNs and ATDs free SLNs were prepared and serially diluted the stock solutions. The wells were bored on the medium using a cork borer having diameters of 8 mm. The 100 µl of each diluted samples were incorporated into each well. The 100 µl of drug-free SLNs were also incorporated into 5 wells of the separate plate and considered as a control group. The growth of *Mycobacterium* around the wells was evaluated after 48 h incubation at 37°C and MICs of pure ATDs, a physical mixture of ATDs and ATDs loaded SLNs were determined.

## 3. Results and Discussion

### 3.1 Particle Size, PDI, zeta potential and morphology determinations

The average particle size and PDI of ATDs loaded SLNs were found to be 195.1 nm and 0.229, respectively (Fig 2). Whereas, the zeta potential of ATDs loaded SLNs was found to be -41.9 mV (Fig 3). The results were seen in a formulation containing lipids, stearic acid and Compritol®888 ATO ratio 1:3 and drug to lipid ratio 1:2 and the equal ratio (1%) surfactant, poloxamer 188 and co-surfactant, sodium taurocholate.

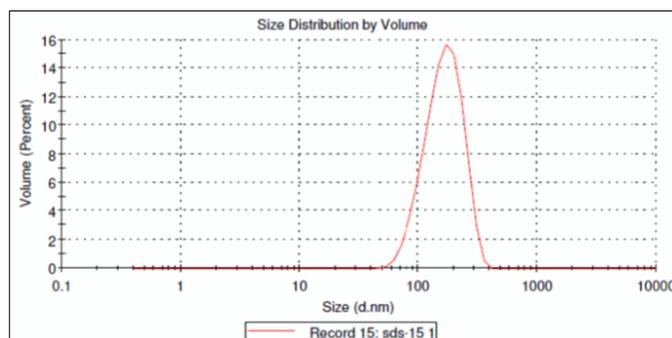


Fig 2: Zeta potential of ATDs loaded SLNs.

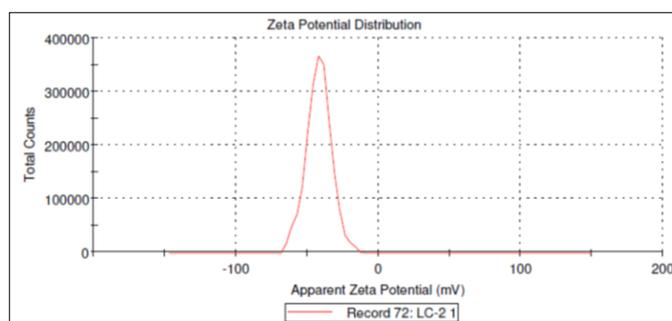


Fig 3: Zeta potential of ATDs loaded SLNs.

### 3.2 Entrapment efficiency, loading capacity and practical yield measurement

Encapsulation efficiency (EE), loading capacity and practical yield are important parameters in respect of the formulation characteristics for the large-scale production. The evaluation of ATDs loaded SLNs for % EE of RIF, INH and PYZ in ATDs loaded SLNs was found to be 84.6%, 83.3% and 76.4%, respectively. The loading capacity was found to be 43.8%, 35.4% and 39.7%, respectively. The SLNs formulation was showed maximum practical yield approx 92.6%, respectively.

### 3.3 Antimycobacterial activity

The ATDs loaded SLNs formulation was analyzed for antibacterial study and found improvement in the antimycobacterial activity (Fig 4). The concentration of pure RIF and INH showed inhibitory action at 388.75 µg/ml and

318.99 µg/ml solutions with 12.7 mm and 16.2 mm zone of inhibition (ZOI), respectively. Whereas, no concentration of PYZ upto 1462.86 µg/ml inhibited the bacterial growth of *M. smegmatis* (Table 1).

**Table 1:** Anti-TB activity of pure ATDs against *M. smegmatis* mc<sup>2</sup> 115

Sample code	RIF		INH		PYZ	
	Conc. (µg/ml)	ZOI (mm)	Conc. (µg/ml)	ZOI (mm)	Conc. (µg/ml)	ZOI (mm)
A	777.50	15.5	637.99	21.0	1462.86	–
B	388.75	12.7	318.99	16.2	731.43	–
C	194.38	–	159.49	–	365.72	–
D	97.19	–	79.75	–	182.86	–
E	48.59	–	39.87	–	91.43	–

ZOI: Zone of Inhibition

The physical mixture of RIF, INH and PYZ (made exactly the same as ATDs loaded SLNs) was (100% solution) having the concentration of 77.5 µg/ml, 64.0 µg/ml and 146.0 µg/ml, respectively showed 12.9 mm ZOI, while ATDs loaded SLNs

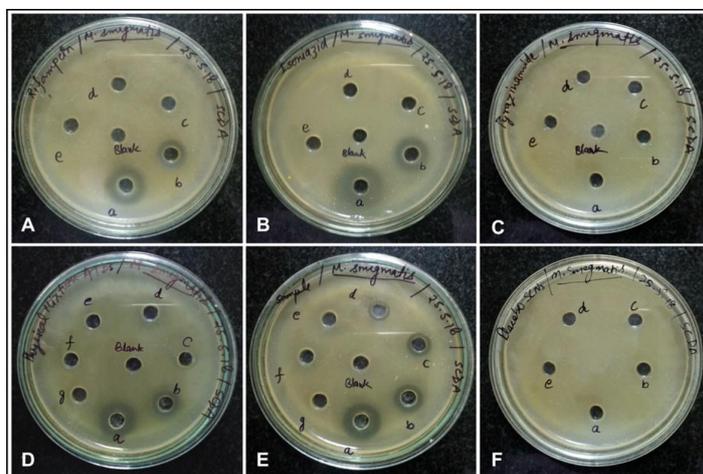
formulation (50% solution) having concentration of RIF, INH and PYZ were 38.75 µg/ml, 32.0 µg/ml and 73.0 µg/ml, respectively showed 9.8 mm ZOI against *M. smegmatis* (Table 2).

**Table 2:** Antitubercular activity physical mixture of ATDs and ATDs loaded SLNs against *M. smegmatis* mc<sup>2</sup> 115

Sample code	Drugs	Concentration (µg/ml)	A physical mixture of ATDs	ATDs loaded SLNs
			Zone of inhibition (mm)	Zone of inhibition (mm)
A (200%)	RIF	155.0	17.6	16.4
	INH	128.0		
	PYZ	292.0		
B (100%)	RIF	77.5	12.9	13.8
	INH	64.0		
	PYZ	146.0		
C (75%)	RIF	58.125	–	12.4
	INH	48.0		
	PYZ	109.5		
D (50%)	RIF	38.75	–	9.8
	INH	32.0		
	PYZ	73.0		
E (25%)	RIF	19.37	–	–
	INH	16.0		
	PYZ	36.5		

These results showed that the ATDs loaded SLNs was approximately ten-times more effective as compared with the standard drugs and showed at least two-times efficacy improvement in comparison to a physical mixture of ATDs solution. Drugs-free SLNs was also prepared similarly to the ATDs loaded SLN formulation except no drugs were used.

Drugs-free SLNs had no effect on the *Mycobacterium* growth. These results indicated that the improved antimycobacterial activity is related to the ability of ATDs incorporated in SLNs. The SLNs formulation was delivered the drugs efficiently to *Mycobacterium* and not to the antimycobacterial effect of any other ingredients in the formulation.



**Fig 4(A):** Zone of inhibition exhibited by RIF solution; (B): Zone of inhibition exhibited by INH solution; (C): Zone of inhibition exhibited by PYZ solution; (D): Zone of inhibition exhibited by Physical mixture of ATDs; (E): Zone of inhibition exhibited by ATDs loaded SLNs (Sample); (F): Zone of inhibition exhibited by ATD free SLNs

#### 4. Conclusion

The microemulsion method used to prepare ATDs loaded SLNs has been found to be very simple and efficacious to produce highly encapsulating nano-sized SLNs. The optimized ATDs loaded SLNs formulations were obtained with an average particle size of  $195.1 \pm 11.11$  nm, may be helpful in prolonging the circulation time of nanoparticles in blood. The PDI value was found to be less than 0.5, desirable for pharmaceutical nanoparticles. The optimized ATDs loaded SLNs formulations carried the negative charge ( $> -30$  mV), were considered as pharmaceutically stable. Encapsulation efficiency, loading capacity and practical yield are important parameters in respect of the formulation characteristics for the large-scale production. The resultant ATDs loaded SLNs formulation showed more than 80% entrapment efficiency and more than 35% loading of ATDs. The SLNs formulation was showed maximum practical yield approx 93%, respectively.

The results of antimycobacterial activity on *M. smegmatis* mc<sup>2</sup> 115 showed that the ATDs loaded SLNs was approx. ten-times more effective as compared with the standard drugs and showed at least two-times efficacy improvement in comparison to a physical mixture of ATDs solution. These results indicated that the improved antimycobacterial activity is related to the ability of ATDs incorporated in SLNs. The SLNs formulation was delivered the drugs efficiently to *Mycobacterium* and not to the antimycobacterial effect of any other ingredients in the formulation.

#### 5. Acknowledgements

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#### 6. Conflict of interest

The authors confirm that this article content has no conflict of interest.

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