Rifampicin dry powder inhalers produced by physical mixing and spraying into antisolvent: stability and biological activity

Somchai Sawatdee, Narumon Changsan, Nimit Worakul and Teerapol Srichana*

Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand, 90112

* Corresponding author

Fax: +6674428148

E-mail address: teerapol.s@psu.ac.th

Abstract

Rifampicin is the first line drug recommended by the World Health Organisation for the treatment of tuberculosis. However, relatively high doses of orally administered drugs are often required to maintain therapeutic concentrations for long periods, which lead to several side effects. To overcome this problem, the delivery of rifampicin directly to the lungs may improve therapeutic efficacy. Rifampicin was formulated by physical mixing with micronised or fine carrier (trehalose, mannose or lactose). Rifampicin was also formulated with trehalose or mannose as a dry powder by spraying into an antisolvent. Drug susceptibility testing by flow cytometry of *Mycobacterium bovis* after incubating with rifampicin formulations and standard rifampicin was performed. We found that rifampicin with fine trehalose, micronised mannose and fine lactose have fine particle fraction over 60%. The different size of carrier particles strongly affects the lower deposition of rifampicin *in vitro*. For spraying into antisolvent technique, the ratio of cholesterol and phosphatidylcholine 1:3 by weight gave the highest encapsulation of rifampicin (95.36 ± 1.43%) and only trehalose showed the suitable physical properties as a carrier for rifampicin dry powder inhalers. The mass median aerodynamic diameter of all formulations varied from 2.66 ± 0.62 to 4.41 ± 0.31 μm. At low concentrations of rifampicin dry powder formulation (0.03-1.5 μg/ml), there are lower viable cells of *M. bovis* than standard rifampicin ($P < 0.05$) however at higher concentrations (3-30 μg/ml) there are no difference between the formulation and pure drug ($P > 0.05$). The stability of dry powder formulations was acceptable in formulations employing aqueous polyethylene glycol as an antisolvent. However, the obtained aerodynamic diameter from these formulations are larger than 5 μm which is ont an ideal size for deep lung penetration.

Keywords : rifampicin, dry powder inhaler, physical mixing, spraying into antisolvent

1. Introduction

Tuberculosis (TB) is one of the most significant infectious diseases affecting the world today. The World Health Organization (WHO) estimates that active cases of TB afflict seven to eight million people annually, and lead up to three million deaths per year (Grange and Zumla, 2002). Furthermore, a person infected with HIV is ten times more likely to develop TB than one who is HIV-negative. Consequently, the spread of HIV is accelerating the rise in TB case rates (Blanc and Nunn, 1999 ; Grange and Zumla, 2002).

The causative organism of TB is the tubercle bacilli, named *Mycobacterium tuberculosis*. The current treatment of pulmonary TB involves prolonged oral administration of high doses of combined antibiotics, which are associated with unwanted side effects and poor compliance (Suarez *et al.*, 2001). These factors cause a rise in multi-drug resistant TB (Panchagnula and Agrawal, 2004).

Most oral antituberculosis drugs presently in use fail to achieve high drug concentration in the lung. A few reports suggest that a high drug concentration in the lung will be obtained after maintaining a high dose oral drug administration for a long period of time (Sharma *et al.*, 2001; Liu *et al.*, 2003 and Tsapis *et al.*, 2003). Clinical management of the disease is limited because of toxic side effects. Moreover, there are problems associated with drug; poor stability and low permeability. Rifampicin is a first line drug recommended by WHO for the treatment of TB. However, low effectiveness of oral rifampicin is still a problem today. The development of rifampicin delivered directly to the lung is one of the most promising ways. As inhaler dosage form usually reduces the

drug dose and as a result it causes less side effect and toxicity. Hence, improvement in patient compliance is expected. Most importantly, giving a high local drug concentration may reduce the duration of treatment and prevent the multi-drug resistance of TB. In addition, it can enter phagocytic cells and kill intracellular microorganisms including the tubercle bacillus (Gürsoy *et al.*, 2004).

There are a few studies of antituberculosis drugs as dry powder formulations for delivery to the lower airway. Reverchon *et al.* (2002) formulated rifampicin as microparticles produced by antisolvent technique. However, rifampicin is sensitive to moisture sorption resulting in degradation and loss of activity. In this study we employed antisolvent technique containing various amount of polyethylene glycol in water and purified water as a control. Physical mixing and spray drying are common practices of powder preparation in delivering particles to the lung via DPIs. Hence we used this technique in couple with antisolvent system to obtain solid particle as a suspension form. From previous studies, the results indicated that the delivery of antituberculosis drug is better than conventional drug delivery systems (Dutt and Khuller, 2001 ; Saurez *et al.*, 2001 ; Sharma *et al.*, 2001 ; Vyas *et al.*, 2004). In this study, we formulated rifampicin as DPIs following three methods; physical mixing, spraying into water antisolvent under pan coater. The emitted dose and MMAD were evaluated. The stability and bioactivity of dry powder formulations were monitored.

2. Materials and Methods

2.1 Materials

Rifampicin was a generous gift from Siam Pharmaceuticals, Research and Development, Bangkok, Thailand. Rifampicin standard, cholesterol from lanolin, D-(+)-lactose monohydrate and L-α-phosphatidylcholine from soybean (lecithin) were purchased from Fluka, Switzerland. D-(+)-trehalose dihydrate, D-(+)-mannose, ammonium molybdate and fluorescein diacetate (FDA) were obtained from Sigma Chemicals (St. Louis, MO, USA). Polyethylene glycol 4000 and sodium dihydrogen phosphate was from Riedel-de Haën, Germany. Chloroform, acetonitrile, methanol, hexane and dimethyl sulfoxide (DMSO) were obtained from J.T. Baker (NJ, USA). All of these solvents were high performance liquid chromatography (HPLC) grade. BCG vaccine was supplied by Aventis Pasteur (Toronto, Ontario, Canada). Middlebrook 7H9 broth and ADC supplements were purchased from Difco™ (Detroit, MI, USA). All other used materials were of analytical grade and used as received. Milli-Q-water (Millipore, Watford, UK) was used in all preparations.

2.2 Analysis of rifampicin in dry powder formulations

Rifampicin was analysed by HPLC. The HPLC system consists of a Waters™ 600 controller and Waters™ 600 pump with a Waters™ 717 plus autosampler equipped with a Waters™ 486 tunable absorbance diode-array detector connected to Waters™ 746 data module (Milford, MA, USA). The microbondapak C18 column (Phenomenex, USA) (250×4 mm i.d., 5 μm) was used in this study. The mobile phases consist of 0.05 M sodium dihydrogen phosphate : acetonitrile (55 : 45, v/v) for analysis of rifampicin. The mobile phase was set at a flow rate of 1 ml/min at ambient temperature. The UV detector

was operated at 254 nm. The injection volume was a 50 μl. Rifampicin dry powder formulations were weighed and dissolved in mobile phase to obtain an appropriate concentration whereas the stock solution of standard rifampicin was dissolved in DMSO and then diluted with the mobile phase. Chromatographic and the experimental conditions were validated. Intra- and inter-day precision, accuracy and linearity of rifampicin were determined by this method in the range of the concentration 1-20 μg/ml before the dry powder formulation development.

2.3 Preparation of rifampicin dry powder formulations

2.3.1 Physical mixing

Rifampicin and carriers (trehalose, mannose or lactose) were dried at 37°C for 12 h in a vacuum oven (Precision Scientific, Inc., Chicago, USA). Rifampicin and each carrier was reduced in size by a grinding mill (Fritsch, Germany) for 3 h to obtain micronised particles (particle size ranges of 1-5 μm). Fine carrier particles (particle size ranges of about 10-20 μm) were prepared by a similar method to micronised particles except the grinding time was reduced to 2 h. The drug (6 g) : carrier (10 g) at ratio 1 : 1.67 was mixed in a a screw cap tube which fixed on V-shape mixer (Superline, Japan) with tape and operated at 50 rpm for 2 h. These powder formulations are equal to 100 doses (16 mg per dose). The compositions of formulations are shown in Table 1. All formulations were stored in a desiccator at room temperature over silica gel until required. The effects of carrier size on deposition of these formulations were studied. The formulations with

the fine particle fraction (FPF) (particles < 6.4 μm) over 60% were chosen for further evaluation.

2.3.2 Spraying into antisolvent

A schematic diagram of spraying into antisolvent technique that was employed in this experiment is shown in Figure 1. At the beginning of each experiment, 2 g of lipid (cholesterol and lecithin 1:1 weight ratio) were dissolved with 20 ml of chloroform. Then, 100 mg of rifampicin was added to this solution and stirred until a clear solution was obtained. 400 ml of water or 10%, 20%, 30% PEG 4000 in water was used as an antisolvent in a 30 cm pan coating (Taiyo, Japan) and the pan was operated at 50 rpm. Then, a solution of the formulation was sprayed through a nozzle (diameter 0.5 mm) (Walther Pilot, Germany) with a peristaltic pump (Watson-Marlow Ltd., UK) at a flow rate 0.5 ml/min. At this point, the liquid solution was delivered through the nozzle and fine droplets of liquid were then formed from the precipitation of solute in the antisolvent. Also, the process was operated continuously until the solution was completed. After that, the pan coating was operated for a further 10 min in order to allow complete evaporation of the chloroform. The suspended particles were collected.

The percentage of drug encapsulation was calculated using the following equation:

% encapsulation

$$= \frac{(\text{Total amount of drug loading} - \text{Amount of unencapsulated drugs}) \times 100}{\text{Total amount of drugs loading}}$$

Unencapsulated rifampicin was assayed by sampling 10 ml of filtrate in the production process. This suspension was filtered through a 0.45 μm polyamide membrane filter and the filtrate was established as unencapsulated drug. Then, the resulting solution was assayed for rifampicin concentration by HPLC.

Formulations 10-19 were studied for the effect of lipid ratio on percent encapsulation (Table 2). Different formulations were produced by varied lipid ratio of cholesterol to lecithin from 1:1, 1:2, 1:3, 2:1 and 3:1 by weight. The ratio of cholesterol: lecithin that gave the highest encapsulation of rifampicin was chosen for further study.

2.4 Particle size distribution measurement

The particle sizes based on volume distributions of micronised rifampicin, sugar carriers and formulations of spraying into antisolvent were measured by laser diffraction (Mastersizer, Malvern, UK) using an independent particle size model fitted with a 100 mm lens and an obscuration was kept at 10%. A small amount of product to be measured was dispersed in water containing 1% w/v of Tween 80. Either micronised or fine carriers (trehalose, mannose or lactose) was dispersed in chloroform containing 1% w/v of Span 80 separately. All suspensions were sonicated in an ultrasonic bath for 10 sec before performing sample measurement. Sample bath was filled with 900 ml of the medium and background signal was measured and then the sample was added and mixed homogeneously with the medium. Each measurement was taken in triplicate.

2.5 *Characterization of particle morphology by scanning electron microscopy*

Morphology of all particles in section 2.3 was achieved by scanning electron microscope (Jeol, Japan). A small amount of each sample was scattered on an aluminium stub, the latter surface covered with clear double-sided adhesive tape. In order to obtain uniformly scattered samples the aluminium stub was tapped gently on its edge with a spatula. The particles were then coated with a 20 nm layer of gold using a sputter coater (SPI supplied, USA) on an argon atmosphere (50 Pa) at 50 mA for 50 s. All micrographs were taken at an acceleration voltage of 10 keV.

2.6 *Characterization of lipid vesicle from the spraying into antisolvent formulation*

The internal structure of the lipid suspension from spraying into antisolvent formulation was determined by transmission electron microscopy (TEM) (Jeol, Japan). The suspension (100 mg) was mixed with 50 mg of uranyl acetate and adjusted the volume with 1 ml of water. Following these, the sample suspension was left at room temperature for about 1 h. After that, this mixture was added dropwise onto a 200 mesh-carbon-grid on a filter paper and then dried at room temperature for about 3 h. The thin film was determined by TEM at 100 keV. In addition, a confocal laser scanning microscopy (FV300, Olympus, Japan) was used for determination of the multilayers of lipid vesicle. The suspension sample was mixed with 1% Nile blue A at the volume ratio about 1:1. Then, the mixture was determined by visualization of fluorescent signals and transmitted light with a confocal laser scanning microscope equipped with an argon laser were recorded.

2.7 Determination of chloroform in the spraying into antisolvent formulation

20 mg of microcapsule obtained from spraying into antisolvent technique was weighed and placed in a 10 ml volumetric flask and adjusted to volume with water. This suspension was sonicated until a clear solution was produced. The chloroform content in the solution was analyzed according to the protocol described by Golfinopoulos *et al.*, (1998) and Kostopoulou *et al.*, (2000). Briefly, Liquid-liquid extraction was used to extract chloroform in the water sample. The chloroform was determined by purge-and-trap gas chromatographic method (Hewlett Packard, HP 7695). One μl of the extract in hexane was then injected into a gas chromatography. The injection technique used on-column and split mode technique. A 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica capillary column Rtx-5MS was used in this study. Helium was used as a carrier and nitrogen as a make-up gas.

2.8 Content uniformity of dry powder formulations

Each formulation was examined by sampling a dry powder formulation following the USP 24 guideline. A total of 10 doses were collected, three doses at the top, four in the middle, and three at the bottom of the bottle containing powder blends. After that, the samples were dissolved and diluted with a mobile phase. All solutions were assayed according to HPLC. The mean actual drug content was expressed as a percentage of the theoretical drug content and a relative standard deviation (RSD) of these values was used to assess the homogeneity of the blends.

2.9 *In vitro deposition of rifampicin dry powder formulations*

The deposition of each dry powder formulation was assessed *in vitro* using an Andersen Mark II cascade impactor (ACI) (Atlanta, Georgia, USA). 16 mg of physical mixing formulation or 20 mg of spraying into antisolvent formulation was weighed and placed in glass inhaler device which was described elsewhere (Srichana *et al.*, 2003). The air flow was drawn through the device at a standard flow rate of 60 l/min for 10 s. This was delivered into ACI and the experiment was carried out 6 times. For each delivery, the powder deposited on the different stages was recovered by washing each plate and the above stage with a mobile phase. The powder deposited in the glass device, inlet and preseparator was also collected in a separated container. All eluents were adjusted to the appropriate volume with the same solvent. The drug concentrations in these samples were assayed by HPLC.

The emitted dose (ED) was determined as a percent of total powder mass exiting the inhaler device. The mass of powder deposited on each stage of the ACI was calculated as a cumulative percentage and plotted against the log size of effective cut-off diameter. The y axis was obtained by transformation of the percent cumulative oversize on various stages of the ACI to a probability scale (z value) and x axis was on logarithm of size. The mass median aerodynamic diameter (MMAD) corresponds to z value of 0 and the geometric standard deviation (GSD) was obtained by the square root of the size of z value of 1 divided by the size obtained at z value of -1.

2.10 Stability of dry powder formulation

All formulations were stored in a desiccator at room temperature. The percentage of content of drugs was determined after storage for 3 months by HPLC. The MMAD was calculated and compared to its initial MMAD.

2.11 Culture of M. bovis

M. bovis (BCG strain) obtained from BCG vaccine was cultured by growing the inoculum in Middlebrook 7H9 broth containing 0.5% glycerol and 10% albumin, dextrose and catalase (ADC) enrichment for 21 days at 37°C, without shaking. To prepare the suspension for inoculation, the cultures were vortexed, left for 30 s to allow the settling of heavy particles then the suspension was diluted to reach a turbidity that matched the optical density of a Mcfarland 1.0 standard.

2.12 Preparation of assay suspensions for flow cytometric analysis

The flow cytometric susceptibility is performed according to Kirk *et al.* (1998a ; 1998b) and Norden *et al.* (1995). Briefly, *M. bovis* suspension which adjusted the turbidity equivalent to McFarland 1.0 standard ($1x10^6$ cell/ml) was used. Some cultures of *M. bovis* are diluted by the addition of M7H9 broth. Once a sufficient quantity of *M. bovis* organisms is obtained, serial dilutions of the samples or standard rifampicin (volume 0.5 ml, concentration 30-0.03 µg/ml) were inoculated with 0.5 ml of 1×10^6 *M. bovis* organisms. Drug-free suspensions of *M. bovis* were also included as a control. The suspensions were then incubated for 24 h at 37°C in the presence of 5% CO_2.

After incubation, 0.2 ml of each assay suspensions was placed in a sterile screw-cap micro-tube containing 0.2 ml of FDA prepared at 500 ng/ml in phosphate buffer saline pH 7.4. Samples were then incubated at 37°C for 30 min before being analyzed using flow cytometer (FACSCalibur, Becton-Dickinson, California, USA) and CellQuest™ software for data acquisition and analysis. Initially, M7H9 medium alone, M7H9 medium containing unstained viable *M. bovis* cells, M7H9 medium containing viable *M. bovis* cells stained with FDA and M7H9 medium containing viable *M. bovis* cells incubated with samples for 24 h and then stained with FDA were detected and differentiated from M7H9 particles by using FDA fluorescence. Live gating was performed on profiles of *M. bovis* during data acquisition to exclude all M7H9 particles. Data were acquired for 50 s to obtain approximately 50,000 counts from gate events. Samples were analyzed by histogram profiles of FDA fluorescence using CellQuest™ software. Gates was established for viable and non-viable mycobacteria on the basis of their incorporation of FDA.

2.13 Statistical analysis

The data were statistically processed to determine the level of significance. A pair *t*-test was performed to compare the MMAD of the formulations at the initial stage and after 3 months. The level of significance was taken as $P < 0.05$. The mean of several data sets of viable *M. bovis* incubated with rifampicin dry powder formulations and standard rifampicin alone were compared using one way ANOVA. The significance was evaluated at 5% probability level ($P < 0.05$).

3. Results and discussion

3.1 Validation

DMSO was chosen for preparation of standard rifampicin as it has been shown to maintain rifampicin stability unlike water and acetonitrile in which rifampicin has limited stability (Gallo and Radaelli, 1976). Intra-day and inter-day precision were established by repeat injections of each analyte at the concentration 1-20 μg/ml. RSD values of rifampicin was acceptable for both precision analysis (Bain *et al.*, 1998).

Accuracy was established by a standard addition method of rifampicin at concentrations between 1-20 μg/ml. The percentages of recovery were 99.9% at all concentrations of rifampicin except the lowest concentrations of rifampicin which were 95%.

Linearity of detector response was established by preparing 5 concentrations of rifampicin (1-20 μg/ml). Rifampicin gave a good linearity with a correlation coefficient (r^2) > 0.99 in these concentration ranges.

3.2 Preparation of rifampicin dry powder formulations

One dose of oral rifampicin is 600 mg. Generally, the dose for inhalation may be reduced to less than 40-80 times as compared to a conventional oral formulation. Hence, the dose of rifampicin in physical mixing DPIs is fixed at 6 mg which had total unit

dosages of 16 mg. The content of rifampicin in spraying into antisolvent formulation was 500 μg from 20 mg of dosage.

Fine particle fractions (FPFs) defined as the amount of drugs recovered from the lower stage varied from 0.65 ± 0.26 mg to 4.71 ± 0.24 mg according to the FPFs varied from 11.7 ± 4.7% to 79.0 ± 1.8% depending on the type and ratio of micronised and fine carriers. The results show that carrier size strongly affected the *in vitro* deposition of drugs. In the case of trehalose carrier and lactose carrier, the formulations containing fine particles (particle size range 10-20 μm) showed the highest fine particle values. In contrast, the formulation containing micronized particles (particle size about 1-5 μm) of a mannose carrier shows the highest FPF value of the rifampicin. This result can be partly explained by the fact that interaction between rifampicin and different type of carriers strongly affects the adhesion force of rifampicin and carriers. The lower depositions of rifampicin are similar to a previous study of Bosquillon *et al.* (2001), which showed that sugars/polyol greatly influenced the *in vitro* deposition of the powders (lactose, trehalose and mannitol presenting the best to poorest behavior in the impactor). But the physical characteristics of those powders, particle size, density, and overall morphology, as visualized by electron microscopy, were relatively similar, suggesting that the differences in respirable fractions rather resulted from differences in powder cohesiveness with the type of excipient. Differences in hygroscopicity of the materials and thereby alteration of capillary forces and/or differences in surface properties may explain those results (Bosquillon *et al.*, 2001). The selected powder blend was Formulations #1, #6 and #7 (see compositions in Table 1).

The effects of cholesterol: lecithin ratio on % encapsulation of formulation prepared by spraying into antisolvent are shown in Table 2. We found that lecithin and cholesterol at a ratio of 3:1 gave the highest %encapsulation (about 95%). Similar results were obtained either using trehalose (formulation no. 10-14) or mannose (Formulations #15-19) as carriers (Table 2). This suggests that the carrier does not affect the %encapsulation of rifampicin because the carrier solution was added after the microcapsules were formed. It was observed that the distance between the spray nozzle and antisolvent is critical to be optimized. If the distance is too far, the spraying droplets are smaller from evaporation while in closer proximity, the larger spray droplets will be obtained.

However, carrier type also influenced the physical characteristics and quality of DPIs. The formulations containing trehalose as carriers have good flow characteristics, bulky, suitable particles size and are easier to freeze dry. In contrast, all those formulations formulated with mannose as carrier have poor flowability and are large in size. The moisture sorption may be a factor involving the aggregation. In addition, the freeze drying process of formulations with mannose carrier took a longer time than the formulations using trehalose as carrier (about 4 days for formulations with mannose and 1day for formulations with trehalose). As a result, the formulations with mannose as carriers are excluded as DPIs and will not be further considered in this study.

The lipid ratio of lecithin and cholesterol of 1:3 was chosen to evaluate for the effect of antisolvent on drug stability. It was found that the efficiency of encapsulation decreased when a higher concentration of PEG in antisolvent was used. The emitted dose also

decreased significantly. From the experiment it was observed that there were amount of rifampicin deposited in lower stage however it cannot be quantified due to very low concentration of rifampicin in the dosage.

3.3 Particle size distribution measurement

The grinding mill process produced micronised rifampicin and micronised carriers with a volume median diameter ranging between 3.35 and 6.04 μm. The fine carriers have a volume median diameter ranging between 15.21 and 17.88 μm. The rifampicin exhibited a particle size less than 5 μm over 99%, suggesting that this material was suitable for use as an inhalation aerosol.

The mean of volume median diameter of spraying into antisolvent formulation is 4.51 ± 0.97 (mean ± SD, n = 3). This particle size can be deposited in the lower airways, so this formulation is also appropriate for DPIs.

3.4 Morphology of dry powder formulations

Scanning electron micrographs show that the particle shape of rifampicin and carriers varied from irregular shapes to spherical shapes (Figure 2A-2I). Figure 2A-2G show the scanning electron micrograph of micronised rifampicin, micronised carriers and fine carriers obtained from the grinding mill. The fine lactose exhibited a irregular shape. Trehalose and mannose particles appeared to be slightly more spherical than lactose. The mannose particles were more symmetrical and clearly rounder than either trehalose or lactose. Many fine particles adhered on the large particles surface. As with micronised

materials, the particles tend to be cohesive and form agglomerates as is apparent from the scanning electron micrographs. There was no readily distinguishable difference in the morphology of these fine particles.

Figure 2H and 2I show the morphology of Formulation #20 which was prepared by spraying into antisolvent. The irregular shape particles shown in may be as a result of carrier crystallization. The spherical particles were found with lower magnification (Figure 2H). Figure 2I shows a scanning electron micrograph of the porous texture of the trehalose cake containing encapsulated rifampicin. The results were similar to the study of Winden *et al.* (1999) who formulated liposome consisting of dipalmitoyl phosphatidylcholine: dipalmitoylphosphatidylglycerol (sodium salt):cholesterol in the ratio of 10 : 1 : 4 freeze-dried in trehalose (3 g carrier/g phospholipid).

Figure 3A shows the lipid layers of formulation sprayed into antisolvent obtained from confocal laser scanning microscope. These particles have a size range between 1-10 μm. Most of particles are about 1-5 μm corresponding with the particle size obtained from laser diffraction. The microencapsulated particle is unilamellar but some particles are multilamellar with a large particle. Transmission electron micrograph (Figure 3B) confirms the microcapsule of this formulation. The lipid vesicles revealed multilamellar forms. It was observed that spheres of rifampicin was crystallized in the lamellar phase of the vesicles.

3.5 *Content uniformity of dry powder formulations*

After blending the carriers with rifampicin, we measured the uniformity of drug content. Table 4 shows the content uniformity of all formulations. The rifampicin contents mixed with trehalose and lactose carriers are 98.9 ± 5.4% and 97.9 ± 4.4% respectively. Whereas, the physical mixing with mannose presents greater variations and lower in drug content (85.2 ± 4.7%). Probably, the mannose itself is very sensitive to moisture.

The content uniformity of formulation #20 is 79.6 ± 2.7% when compared with the theoretical content. Since the spraying into antisolvent is an open system, the suspension was lost from the coating pan. From Table 4, all the rifampicin formulations showed rapid decrease in drug content when the products was kept for 3 months since rifampicin is sensitive to moisture and light. Solid formulation of rifampicin was slower to degrade than in a solution formulation. It was found that rifampicin was stable over three months in a formulation of spraying into PEG antisolvent. It is postulated that PEG may reduce the water activity against rifampicin resulting in more stable form of rifampicin. Hence this approach should be further inverstigated because still the aerosolized properties were poor.

To ensure that chloroform was completely evaporated from the formulation, the chloroform content was determined. The result shows that chloroform content of this formulation was not detected (the limit of detection is 0.25 ng/ml). Therefore, it can be ensured that the formulation is safe for inhalation.

3.6 In vitro deposition of rifampicin to lower airway

Figure 4 depicts the particle size distribution of a single dose delivery from selected rifampicin DPI Formulations #1, #6, #7 and #20. Each bar represents the powder of certain sizes collected on a defined stage of the ACI. These results show the weight fraction according to the size distribution of the aerosolized particles of rifampicin with a different type and ratio of micronised and fine carriers in physical mixing formulation and formulation sprayed into antisolvent.

The ED of physical mixing formulations varied from 5.80 to 5.98 mg (nominal dose is 6 mg) when calculated from ACI except the ED of spraying into antisolvent formulation is 95.0 % (nominal dose is 500 μg). The size distribution of Formulation #1, #6 and #7 is similar in their pattern. The results suggest that the physical interaction of rifampicin and fine trehalose, micronised mannose or fine lactose is suitable for the delivery of rifampicin to the lower airways and can be deposited on stages 4-7 of the ACI (Figure 4) which predicted to be an alveoli. The size distribution of Formulation #20 was different from physical mixing formulations. The drug in this formulation traveled as far as stage 7 which was better than the formulation prepared by physical mixing. FPF of selected formulations are shown in Table 1. These selected formulations deposited in lower airway over 60%. GSD were between of 1.54 to 1.98 therefore these formulations are polydisperse.

The average MMAD of each powder formulation represents in Table 4. The MMAD varies from 2.66 ± 0.62 μm to 4.41 ± 0.31 μm according to aerosol powders ranging

from 1 to 5 μm are considered as the optimum size for deposition beyond the increasingly narrow airway in the alveoli. The MMAD of the initial formulations was compared with the formulations which were storaged after 3 months. The physical mixing formulations of rifampicin containing fine lactose and rifampicin spraying into antisolvent formulation showed MMAD values after storage are significantly larger than the initial formulations ($P < 0.05$). The significant increase of MMAD after storage is probably due to the formation of particle aggregates. Therefore, these formulations should be improved in order to prevent the aggregation of particles. In contrast, other formulations showed that the MMAD value after storage showed no significant difference ($P > 0.05$). Although spraying into antisolvent formulations were stable but their MMAD were larger than the appropriate values for inhalation.

3.7 Drug susceptibility testing

The method was based on the ability of viable *M. bovis* organisms to hydrolyze FDA to free fluorescein with detection of fluorescent mycobacteria by flow cytometric analysis. It is known that FDA is nonpolar, non fluorescent molecule capable of diffusing across the cell wall and cell membranes of mycobacteria and other bacteria by active transport and passive diffusion (Norden *et al.*, 1995). Once in the cytoplasm, FDA is rapidly (in 5 min) hydrolyzed by esterases to fluorescein. Metabolically inactive and non-viable bacteria have decreased quantities of active esterases that result in these organisms demonstrating less fluorescein.

The M7H9 medium containing viable *M. bovis* cells incubated with rifampicin for 24 h and then stained with FDA produced a histogram profile of organism. Viable *M. bovis* cells were detected in the M7H9 medium although the cells were unstained. *M. bovis* organisms incubated with FDA, only viable *M. bovis* organisms demonstrated a fluorescent intensity, with a mean channel fluorescence of 1538. Unstained *M.bovis* or non-viable *M. bovis* organisms did not hydrolyze FDA and had a mean channel fluorescence of approximately 2.68. When these experiments were repeated, similar results were obtained. The mean channel fluorescence correlated with viability or non-viability of mycobacteria organism (Norden *et al.*, 1995). In addition, when the mean channel fluoresescence was determined after 48 h of incubation, similar results were obtained as incubation for 24 h (Kirk *et al.*, 1998a). We used the viable cell of *M. bovis* after incubation with rifampicin dry powder formulations compared with rifampicin alone to determine the efficacy of drug.

Kirk *et al.* (1998a) suggested that multiplication occurred in some assay suspensions but multiplication was not necessary to discern differences between drug-free suspensions of mycobacteria and those treated with antimycobacterial agents. Figure 5 shows the viable *M bovis* cells after incubation with the rifampicin dry powder formulations and standard rifampicin.

At low concentrations of rifampicin (0.03-1.5 μg/ml) viable cells of *M. bovis* organism of all formulations are lower than standard rifampicin ($P < 0.05$) because the dry powder formulations killed mycobacteria more effectively than standard rifampicin. At higher concentrations (3-30 μg/ml), the viable cells of *M. bovis* incubated with these formulations are not different from standard rifampicin ($P < 0.05$).

4. Conclusions

Physical mixing of rifampicin with carriers (trehalose, mannose or lactose) can be used to produce dry powder aerosols. Dry powder formulations are suitable for lung delivery of rifampicin. Rifampicin can be encapsulated in cholesterol and lecithin vesicle by spraying into antisolvent technique. However, this experiment cannot produce a suitable size for lung delivery after evaluation *in vitro*. Therefore, it is necessary to improve aerosolized properties of inhalation formulation. All formulations have shown antimycobacterial activity against *M. bovis* BCG when compared to standard rifampicin. This may indicate that the activity in the formulations does not change from standard rifampicin.

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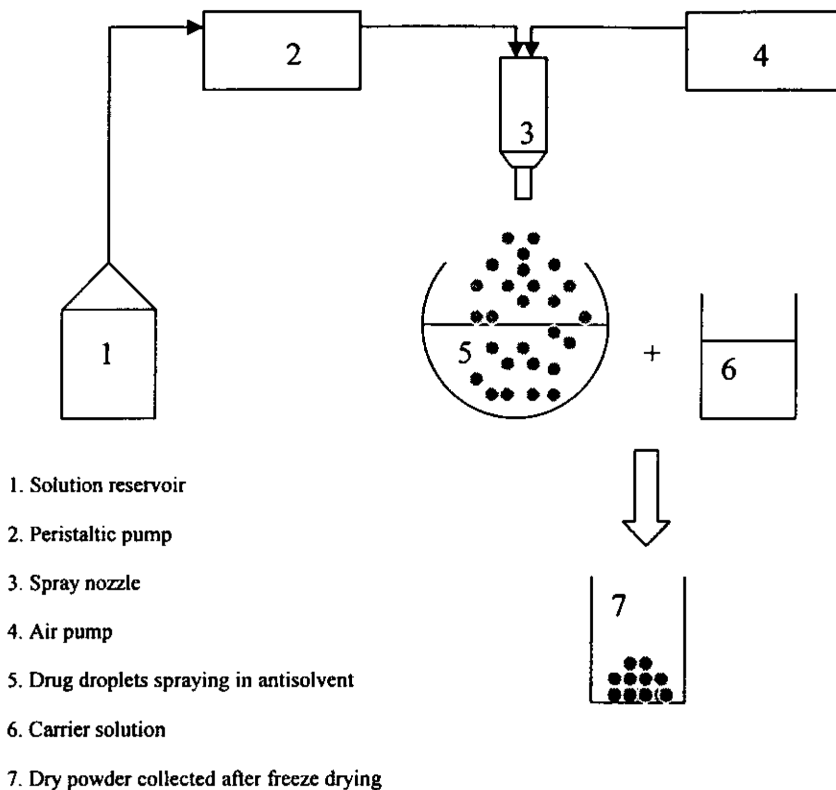

Fig. 1. Schematic diagram of the spraying into antisolvent process.

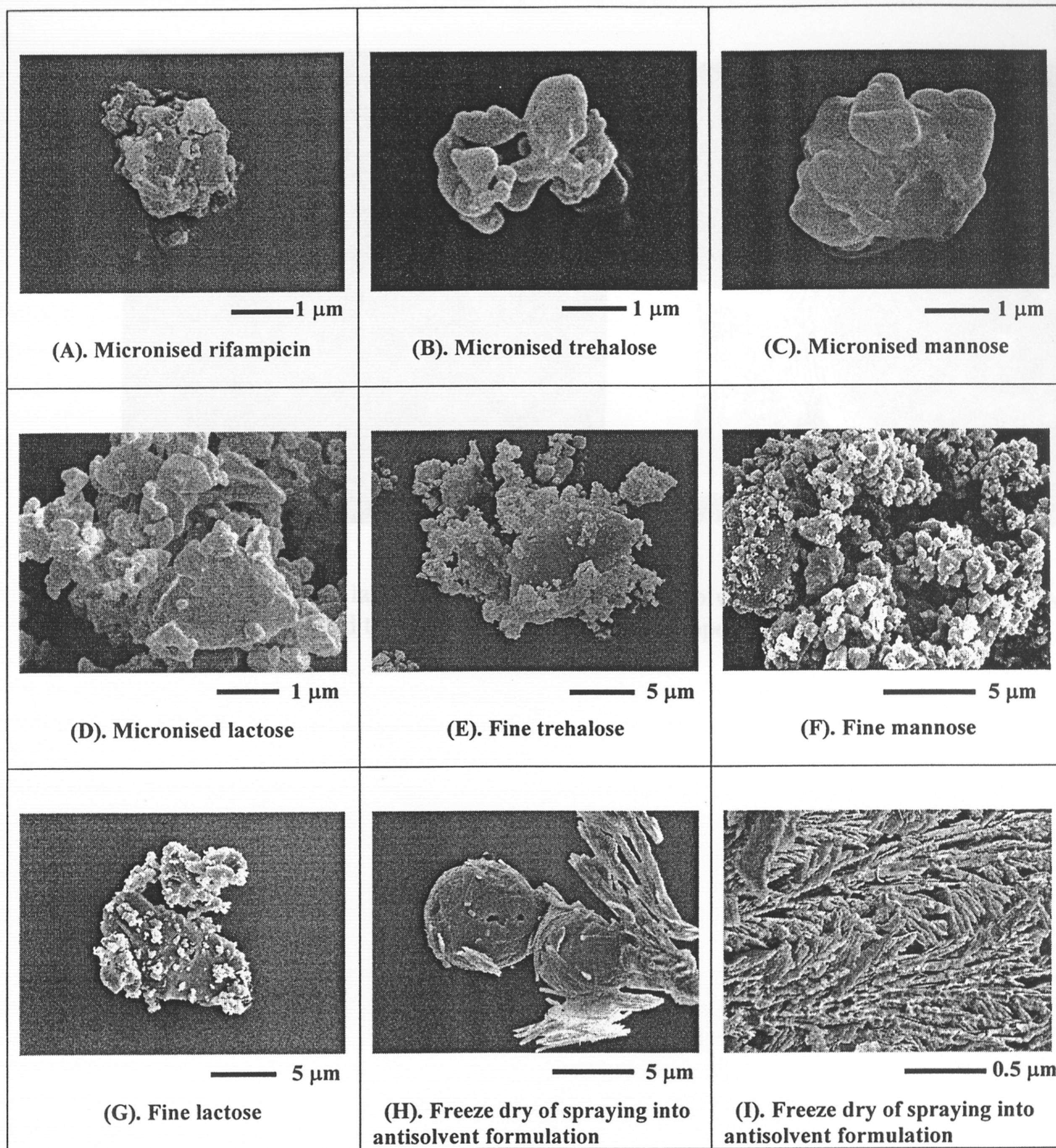

Fig. 2. Scanning electron micrographs of micronised rifampicin, micronised and fine carriers and spraying into antisolvent formulation.

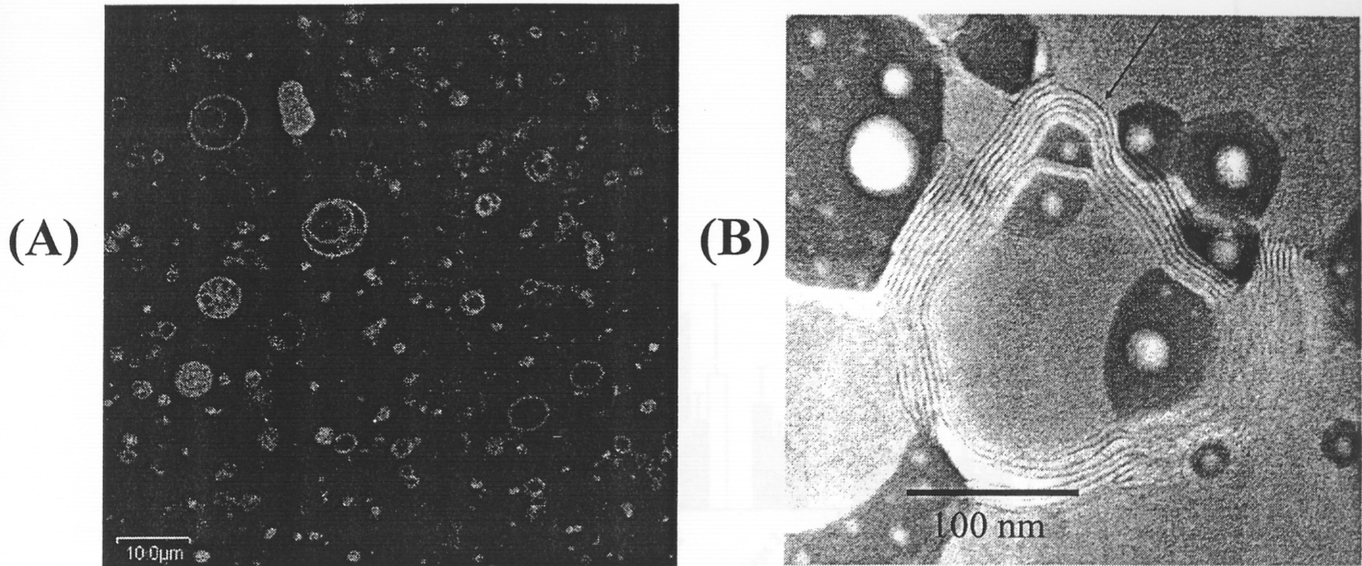

Fig. 3. Photographs of lipid layers of the spraying into antisolvent obtained from confocal laser scanning microscope (A) and transmission electron microscope (B).

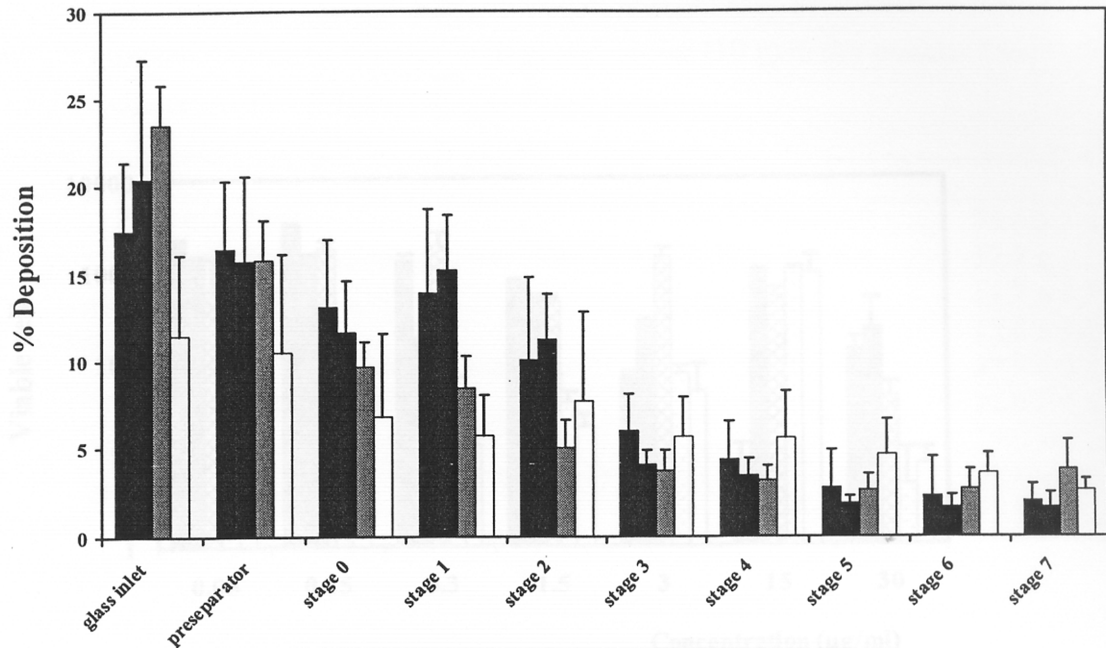

Fig. 4. Size distribution on each stage of the ACI as aerosolized at a flow rate of 60 l/min of rifampicin formulations obtained from physical mixing with fine trehalose (■), physical mixing with micronised mannose (▨), physical mixing with fine lactose (⊠) and rifampicin formulation obtained from spraying into antisovent with trehalose (□) (mean ± SD, n = 6).

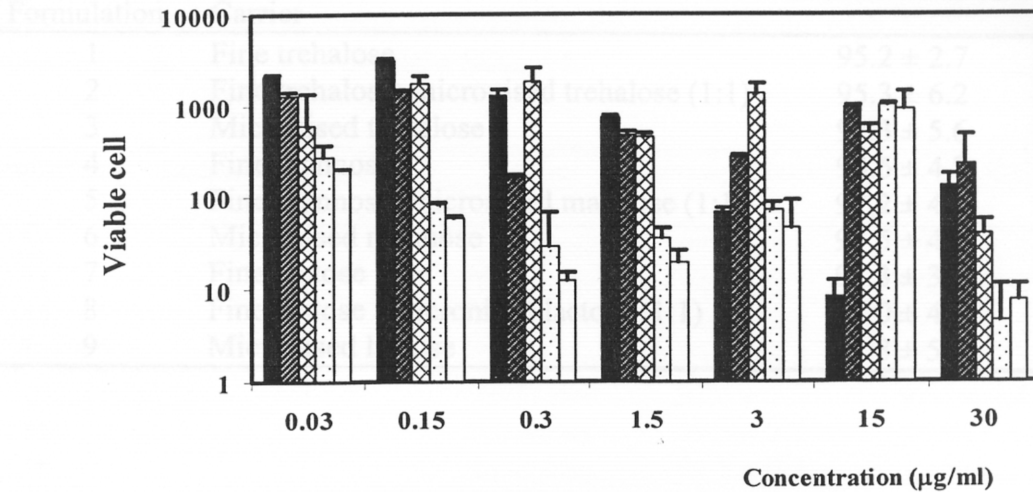

Fig. 5. Viable *M. bovis* cells after incubated with standard rifampicin, (■), physical mixing with fine trehalose (▨), physical mixing with micronised mannose (⊠), physical mixing with fine lactose (⬚) and rifampicin microcapsule formulation (□). All formulations were incubated for 24 h and then stained with fluorescein diacetate and detected by flow cytometry.

Table 1.

Compositions of physical mixing of rifampicin (6 g) and carrier (10 g) in dry powder formulations and aerosolization characteristics (Mean ± SD, n= 6)

Formulation	Carrier		
1	Fine trehalose	95.2 ± 2.7	75.4 ± 3.2
2	Fine trehalose: micronised trehalose (1:1)	95.3 ± 6.2	57.7 ± 4.7
3	Micronised trehalose	92.3 ± 5.6	11.7 ± 4.7
4	Fine mannose	94.6 ± 4.8	13.6 ± 5.2
5	Fine mannose: micronised mannose (1:1)	97.2 ± 4.2	37.7 ± 3.3
6	Micronised mannose	97.5 ± 4.0	72.9 ± 4.9
7	Fine lactose	97.3 ± 3.0	79.0 ± 1.8
8	Fine lactose : micronised lactose (1:1)	97.0 ± 4.8	52.1 ± 0.8
9	Micronised lactose	95.8 ± 5.6	17.2 ± 1.5

Table 2.

Compositions and percent encapsulation of rifampicin into various lipid ratios (soy lecithin and cholesterol) 1:20 after spraying into antisolvent formulations with lipid-carrier ratio 1:1 and aerosolization characteristics (Mean ± SD, n= 6)

Formulation	Lecithin: cholesterol	Carrier	%encapsulation	% emitted dose	%FPF
10	1:1	Trehalose	94.5 ± 1.4	95.4 ± 1.4	UD
11	2:1	Trehalose	94.2 ± 0.5	96.7 ± 2.3	UD
12	3:1	Trehalose	95.4 ± 1.4	95.6 ± 1.6	UD
13	1:2	Trehalose	93.8 ± 0.5	95.0 ± 1.3	UD
14	1:3	Trehalose	91.4 ± 1.0	92.7 ± 1.4	UD
15	1:1	Mannose	89.3 ± 2.7	ND	ND
16	2:1	Mannose	91.6 ± 0.3	ND	ND
17	3:1	Mannose	93.5 ± 0.5	ND	ND
18	1:2	Mannose	90.6 ± 1.0	ND	ND
19	1:3	Mannose	90.2 ± 1.0	ND	ND

ND = Not determined

UD = unable to determine

Table 3.

Percent encapsulation and emitted dose of rifampicin formulation (drug loading 1:20) containing soy lecithin and cholesterol (3:1) after spraying into aqueous antisolvent water and polyethylene glycol (PEG) and trehalose was employed as a carrier (Mean ± SD, n= 6)

Antisolvent	Formulation	Rifampicin (mg)	%encapsulation	% emitted dose	%FPF
water	20	10	94.5 ± 1.4	95.0 ± 1.5	UD
10% PEG	21	10	91.3 ± 6.4	95.2 ±1.3	UD
20% PEG	22	10	80.4 ± 1.1	82.8 ±1.1	UD
30% PEG	23	10	73.3 ± 2.7	72.3 ±1.3	UD

UD = unable to determine

Table 4

Percentage content of drug and MMAD of rifampicin of various formulations at initial and after three months storage (mean ± SD, n=6)

Formulation	%Content		MMAD (μm)	
	Initial	After 3 months	Initial	After 3 months
1	98.9 ± 5.4	84.2 ± 4.3	4.28±0.74	4.94±0.47
7	85.2 ± 4.7	66.5 ± 4.9	4.41±0.31	5.14±0.65
8	97.9 ± 4.4	88.5 ± 1.6	3.67±0.74	4.94±0.47[a]
20	79.6 ± 2.6	67.5 ± 2.7	2.66±0.62	6.23±1.14[a]
21	99.2 ± 1.5	98.2 ± 1.1	> 1.4	> 1.4
22	100.1 ± 1.2	99.8 ± 2.1	> 1.4	> 1.4
23	99.3 ± 1.6	99.4 ± 3.8	> 1.4	> 1.4

[a] MMAD after 3 months was larger than initial (*P* value < 0.05)