Preparation of isoniazid as dry powder formulations for inhalation by physical mixing and spray drying

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Abstract

The main purpose of this study is to develop isoniazid as dry powder aerosol for delivery to the lower airways and to study the susceptibility of *M. bovis* and *M. tuberculosis* to the formulations studied. Isoniazid was formulated with trehalose, mannose and lactose by physical mixing and spray drying techniques. All formulations were evaluated for delivery efficiency and stability. Susceptibility tests of Mycobacterium species to the drug formulations were carried out. Isoniazid mixed with fine trehalose, micronised mannose or fine lactose produced the formulations which gave fine particle fraction (< 5 μm) of over 60%. The different size of carrier particles strongly affects the deposition of isoniazid to the lower airways *in vitro*. The content of all isoniazid dry powder formulations was almost 100% of the theoretical content and found to be stable over 3 months storage. These formulations showed that their mass median aerodynamic diameter varied from 3.14 ± 0.38 to 4.40 ± 0.65 μm. Drug susceptibility testing of *M. tuberculosis* by broth microdilution showed that the MIC of isoniazid dry powder formulations were 1.7 to 3.4 times lower than standard isoniazid. Flow cytometry analysis of viable *M. bovis* revealed that MIC of isoniazid dry powder formulations and standard isoniazid had no significant difference ($P > 0.05$).

Keywords: isoniazid, dry powder inhaler, physical mixing, spray drying, flow cytometry

1. Introduction

Tuberculosis (TB) is one of the most significant infections causing disease in man. Although the incidence of TB declined greatly during the twentieth century, it has become a major problem in human immuno-deficiency virus (HIV) patients. 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 (Reichman and Hershfield, 2000). 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; Reichman and Hershfield, 2000).

The causative organism of TB is 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). As a result, it causes a rise in multi-drug resistant TB (Panchagnula and Agrawal, 2004). Effective chemotherapy of TB involved daily administration of one or more drugs for a period of six months or longer. The initial intensive phase of two months requires four drugs including isoniazid, rifampicin, pyrazinamide and streptomycin or ethambutol. The continuation phase can be four to six months, during which rifampicin is combined with isoniazid. However, the adherence to full treatment is difficult from drug intolerance and many patients stop treatment before completing the regimen (Blanc and Nunn, 1999; Deol and Khuller, 1997).

Most oral antituberculosis drugs presently in use fail to achieve high drug concentration in the lung. A few reports suggest that 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; Tsapis et al., 2003). Clinical management of the disease is difficult because of toxic side effects. Moreover, there are problems caused by the degradation of drugs before reaching their target site and poor patient compliance.

The development of antituberculosis formulation which can be delivered directly to the lung is one of the most promising ways. It is expected that antituberculosis inhalers can reduce the dose and frequency of administration resulting in less toxicity and improvement in patient compliance. Most importantly, targeting the drug to the alveolar macrophage may improve the efficacy and potentially reduce the systemic toxicity of the drug. The high drug concentration localized in the lung may reduce the duration of treatment and prevent the multi-drug resistance of TB.

Physical mixing and spray drying are common methods for preparing powder preparations for delivery to the lung via dry powder inhalers. However, there are very few studies of antituberculosis drugs as dry powder formulations for delivery to the lower airways (Sharma et al., 2001; Suarez et al., 2001; Tsapis et al. 2003). A therapeutically effective amount of an active pharmaceutical preparation will vary in composition depending on the biological activity of the drug employed and the amount needed in a unit dosage form. If the compounds are dispersible, it is highly preferred that they are manufactured in a unit dosage form in a manner that allows for ready

manipulation by the formulator and the consumer. This generally means that the total content of materials in the powder of a unit dosage will be between 0.5 and 15 mg, preferably between 2 and 10 mg. The amount of a pharmaceutically acceptable carrier is the amount needed to provide the necessary stability, dispersibility, consistency and bulking characteristics to ensure a uniform pulmonary delivery of the composition. Generally, the amount of drug in the composition will vary from 0.05 to 99.0% w/w (Platz et al., 2003).

Spherical particles can be prepared by spray drying. Spray drying is a process in which a homogeneous aqueous mixture of a drug and carrier is atomized via a nozzle and introduced to a hot gas. Fine particles will be obtained. The aqueous mixture may be a solution or suspension but needs to be homogeneous to ensure uniform distribution of components in the mixture and ultimately the powdered composition. The solvent (generally water) evaporates rapidly from the droplets producing fine dry powder 1 to 5 μm in diameter (Platz et al., 2003). The shape, morphology and density of the powder particles depend on the spray drying conditions (Steckel and Brandes, 2004). Spray drying technique was used to formulate large porous particles of *para*-aminosalicylic acid for delivery into rat lungs via inhalation (Tsapis et al., 2003).

Results had indicated that the delivery of antituberculosis drug via inhalers direct to the lung is better than conventional drug delivery systems from studies by Dutt and Khuller, 2001; Suarez et al., 2001; Sharma et al., 2001; Vyas et al., 2004. In this study, we formulated isoniazid as dry powder inhaler and evaluated the deposition *in vitro*. *In vitro* susceptibility studies were also carried out.

2. Materials and Methods

2.1 Materials

Isoniazid, D-(+)-trehalose dihydrate, D-(+)-mannose and fluorescein diacetate (FDA) were obtained from Sigma chemical company (St. Louis, MO, USA). D-(+)-lactose monohydrate was purchased from Fluka, Switzerland. Sodium dihydrogen phosphate was purchased from Riedel-de Haën, Germany and chloroform was purchased from VWR International Ltd., UK. Acetonitrile, methanol and hexane (95%) were obtained from J.T. Baker (NJ, USA). All these solvents were HPLC grade. BCG vaccine was supplied by Aventis Pasteur (Toronto, Ontario, Canada). Middlebrook 7H9 broth and other supplements were purchased from Difco™ (Detroit, MI, USA). All other materials used were of analytical grade and used as received. Milli-Q-water (Millipore, Watford, UK) was used in all preparations.

2.2 Analysis of isoniazid in dry powder formulations

Isoniazid was analysed by using 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 (97 : 3 v/v). The mobile phase was set at a flow rate of 1 ml/min at ambient temperature. The UV detector was operated at 254 nm. Isoniazid dry powder formulations and standard isoniazid were weighed, dissolved

and diluted with water to concentrations of 2 to 10 μg/ml before injecting 50 μl into the instrument. Chromatographic and experimental conditions were validated. Intra- and inter-day precision, accuracy and linearity of calibration curve for isoniazid were determined.

2.3 Preparation of isoniazid dry powder formulations

2.3.1 Physical mixing

Isoniazid and the sugar used as carriers (trehalose, mannose and lactose) were dried at 37°C for 12 h in a vacuum oven (Precision Scientific, Inc., Chicago, USA). The size of the drug and carriers were reduced 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 similar method but the grinding time was reduced to 2 h. Before the formulations were prepared, the particles size of isoniazid and all carriers were measured to make sure they were within the expected range (see section 2.4). The 1: 1.67 (w/w) isoniazid : carrier formulations were prepared by mixing 0.6 g of isoniazid separately with 1.0 g of each carrier (trehalose, mannose and lactose) in screw cap tubes for 5 min at room temperature. Each of these mixtures is 100 doses (16 mg per dose). The tubes containing the formulation were fixed on a V-shape mixer (Superline, Japan) with cellophane tape and rotated at 50 rpm for 2 h. All formulations were stored in a desiccator at room temperature over silica gel until required for further use. The details of the formulations are shown in Table 1. The effects of carrier size on deposition *in vitro* of these formulations were carried out as

described in section 2.7. Some formulations were selected for further evaluation if the fine particle fraction (FPF) (particles smaller than 6.4 μm) was over 60% after evaluation by twin stage impinger (TSI) (Copley instrument, Nottingham, UK).

2.3.2 Spray drying

The formulations for spray drying were prepared by dissolving 7.5 g of isoniazid and 12.5 g of the sugar used as carrier (trehalose, mannose and lactose) in 1000 ml of water. The three solutions were immediately sprayed at a flow rate of 10 ml/min utilizing a spray dryer system (Anhydro, Copenhagen, Denmark) equipped with a nozzle of 0.2 mm for atomization. Spray dried powder formulations were collected via a cyclone. This spray condition was optimized beforehand to obtain the particle size less than 5 μm. The isoniazid: carrier ratio was similar to the physical mixing formulations (1:1.67 w/w). The obtained powder formulations were stored over silica gel in a desiccator at room temperature. The compositions of formulations are summarized in Table 1.

2.4 Particle size distribution measurement

The particle sizes based on volume distributions of micronised isoniazid, sugar carriers and spray dried formulations were measured by laser diffraction fitted with a 100 mm lens (Mastersizer, Malvern, UK) using an independent model and an obscuration kept at 10%. Isoniazid and spray dried formulations were dispersed in hexane containing 1% w/v of Span 80. Micronised and fine carriers (trehalose, mannose or lactose) were dispersed in chloroform containing 1% w/v of Span 80. The suspension was sonicated

in an ultrasonic bath for 1 min before performing sample measurements. A background measurement was taken before the sample was added, mixed homogeneously with the medium and the size distribution was measured. Each measurement was taken in triplicate.

2.5 Content uniformity of dry powder formulations

The sampling of each dry powder formulation was carried out according to the USP 24 guideline. A total of 10 sample doses were collected; three doses at the top, four in the middle, and three at the bottom of the bottle containing powder blends. The samples were dissolved and diluted with water and assayed according to the method described in section 2.2. The mean actual drug content was expressed as a percentage of the theoretical drug content. The percentage of relative standard deviation (RSD) of these values was used to assess the homogeneity of the blends.

2.6 Characterization of particle morphology by scanning electron microscopy

Morphology of sampled particles from all formulations were obtained by using scanning electron microscope (Jeol, Japan). A small amount of each sample was scattered on an aluminium stub which surface was 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 15 to 20 nm layer of gold using a sputter coater (SPI supplied, USA) under an argon atmosphere

(50 Pa) at 50 mA for 50 s. All micrographs were taken at an acceleration voltage of 15 keV.

2.7 In vitro deposition of isoniazid 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). Some of the physical mixing and spray dried formulations examined by the TSI were selected and assessed by the ACI. 16 mg of each powder formulation selected was weighed and placed in a glass inhaler device similar to the one used by Srichana et al., 2003. The air flow was drawn through the device at a standard flow rate of 60 l/min into the ACI for 10 s. The experiment was repeated 6 times. For each delivery, the powder deposited on different stages was recovered by washing each plate and the above stage with water. The powder remaining in the glass device and that deposited on an inlet and preseparator of the ACI were also collected in a separate container. All eluents were adjusted to the appropriate volume with the same solvent. The drug concentrations in these samples were assayed by HPLC as describe previously.

The emitted dose (ED) was determined as the percent of total powder mass exiting the inhaler device. The mass of powder deposited on each stage of the Andersen impactor was calculated as a percentage and plotted against the log (effective cut-off diameter). The y axis was obtained by transformation of the percent cumulative oversize on various stages of the ACI in probability scale and x axis was in logarithm of size. The mass median aerodynamic diameter (MMAD) corresponds to size at 50% cumulative.

The geometric standard deviation (GSD) were obtained by the square root of the size at 84% cumulative divided by the 16% cumulative.

2.8 Stability of dry powder formulation

All formulations were stored in a desiccator at room temperature for 3 months. The percentage of drug contents and the deposition in the ACI were determined. The MMAD was calculated and compared to its initial MMAD.

2.9 Drug susceptibility testing by flow cytometric method

M. bovis (BCG strain) was cultured from BCG vaccine. The culture suspensions were prepared by growing the inoculum in Middlebrook 7H9 (M7H9) broth containing 0.5% glycerol, 10% oleic acid, albumin, dextrose and catalase (OADC) enrichment for 21 days at 37°C, without shaking. To prepare the suspension for inoculation, the cultures were vortexed and left for 30 s to allow the settling of heavy particles. The suspension was diluted to reach a turbidity that matched the optical density of Mcfarland 1.0 standard. The flow cytometric susceptibility was performed according to method described by Kirk et al. (1998a; 1998b) and Norden et al. (1995). Briefly, cultures of *M. bovis* were diluted by the addition of Middlebrook 7H9 broth. Once sufficient *M. bovis* organisms were obtained, serial dilutions of the formulations and standard isoniazid (volume 0.5 ml, concentration 30-0.03 μg/ml) were inoculated with 0.5 ml of 1×10^6 *M. bovis* organisms (Mcfarland 1.0). Drug-free suspensions of *M. bovis* organisms 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 suspension was placed in a sterile screw-cap micro-tube containing 0.2 ml of FDA prepared at 500 ng/ml in phosphate buffered saline at 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, then 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 a formulation for 24 h and then stained with the FDA. *M. bovis* cells were differentiated from M7H9 particles. The viable cells were also detected at different gate event from non-viable cells. 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 by using CellQuest™ software. Gates were established for viable and non-viable mycobacteria on the basis of their incorporation of FDA. This 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 a nonpolar, non fluorescent molecule capable of diffusing across the cell wall and cell membranes of mycobacteria 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.

Whereas non-viable bacteria have decreased quantities of active esterases thus resulting in less production of fluorescein.

2.10 Drug susceptibility testing of M. tuberculosis by the broth microdilution method

The broth microdilution method was performed according to previous study by Coban et al., 2004 and Leite et al., 2000. Briefly, 96-well microtitre plates with U-shaped wells were used in this study. The plates were arranged to give 12 rows by eight lanes and these were filled with 0.1 ml of M7H9 broth, supplemented with 0.1 ml OADC enrichment. All of the selected formulations and standard isoniazid were weighed and dissolved in water to concentrations of 60 µg/ml and their two fold dilutions. 0.1 ml volumes of each formulations and standard isoniazid solutions prepared were dispensed into the wells of the plates. Plates were stored at -20°C until use. Each well was inoculated with 5 µl of McFarland 1.0 standard of *M. tuberculosis* (H37Rv) suspension. A well without antimycobacterial agents was also inoculated with 10^{-2} dilution of McFarland 1.0 standard as a growth control. The plates were sealed, placed in plastic bags and incubated at 37°C for 21 days in an incubator. MIC was defined as a lowest drug concentration that exhibited no growth by microplate reader, and the strains were considered susceptible to each formulation, if their MICs were below or equal to the critical concentration.

2.11 Statistical analysis

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 set at $P < 0.05$. The mean of several data sets of viable *M. bovis* incubated with solutions prepared from isoniazid dry powder formulations and standard isoniazid were compared using one way ANOVA. The significance was at $P < 0.05$.

3. Results and discussion

3.1 Validation

RSD values of intra-day and inter-day analysis of isoniazid were less than 2% (Table 2). The values were acceptable precision for the *in vitro* study (Bain et al., 1998). The percentages of recovery are almost 100% at all concentrations which indicate the accuracy of analytical method (Table 2). The standard curve for isoniazid has a good linearity with a correlation coefficient (r^2) > 0.999 in concentration ranges of 2-10 μg/ml (Figure 1).

3.2 Preparation of isoniazid dry powder formulations

Fine particle doses (FPDs) is defined as the amount of drugs recovered from the lower stage of the TSI. Their values varied from 0.71 ± 0.18 mg to 4.52 ± 0.32 mg which might be related to the FPFs, whose values varied from 11.99 ± 2.87% to 81.50 ± 4.86% depending on the type of carriers. These three sugars are commonly used as carrier in dry powder inhaler (Hickey, 1992). The results show that carrier sizes were strongly affected the *in vitro* deposition of drugs. The formulations containing fine particles of

trehalose, lactose or micronized mannose gave very high FPF (>60%). According to the study of Bosquillon et al. (2001), the type of sugars/polyol greatly influenced the *in vitro* deposition of the powders. In this study particle size and density of these carriers were relatively similar, the differences in FPF observed could have resulted from the type of carrier used. The formulations selected for further evaluation were INH-1 (A), INH-2 (C) and INH-3 (A) (see compositions in Table 1) since they have high FPF (> 60%) (Table 2).

In spray drying, the drying air temperature can be relatively high (> 100°C), the actual temperature of the evaporating droplets is significantly lower due to the cooling effect by the latent heat of vaporization. No thermal degradation of isoniazid was observed in the HPLC chromatogram (data not shown). This is in line with the previous studies (Chan and Chew, 2003). The spray dried formulation no. 11 of isoniazid with mannose was excluded from this study because this formulation turned to melt when the temperature of evaporation increased. This is due to the low glass transition temperature (T_g) of mannose (30°C) (Cortes and Caekenberghe, 1997).

3.3 Particle size distribution measurement

The grinding mill process produced micronised isoniazid and micronised sugar carriers with a volume median diameter (VMD) ranging between 4.07 and 6.04 μm, respectively (Table3). Particle size of isoniazid less than 5 μm (>99%) (Table 3) is suitable for use

as dry powder inhaler. The fine carriers have a VMD ranging between 15.21 and 17.88 μm when using a shorter grinding time than that of micronised sugar carriers (Table3).

Pharmaceutical powders less than 5 μm are predicted to be able to enter the lower airways (Hickey, 1992). Micronised isoniazid has a particle size in the range of 1-5 μm but carriers have two size ranges (1-5 μm and 15-20 μm). Both size ranges can be used to prepare the dry powder formulations with a micronised drug. Reducing the carrier particle size has also been exploited in an attempt to improve *in vitro* drug deposition (Tee et al., 2000). A more practical approach to improve the delivery efficiency of dry powder inhaler, might involve manipulation of the powder formulation. Such an approach could include the addition of fine particles of a third component or micronised carrier to powder formulations (Tee et al., 2000).

In spray drying technique, the mean of the VMD of INH-1 (sd) and INH-3 (sd) was 6.48 ± 1.50 μm and 6.08 ± 0.81 μm (mean ± SD, n = 3), respectively (Table 3). Large particle size was obtained by spray drying technique when compared with grinding mill. Hence, this technique has to be optimized to obtain particle smaller than 5 μm in order to meet the requirement of inhaled aerosols.

3.4 Content uniformity of dry powder formulations

After blending the carriers with isoniazid, we measured the average content and the uniformity of the drug. Table 3 shows the content of isoniazid in all formulations in

percentage. All isoniazid formulations has high content uniformity. The average isoniazid contents of the INH-1 (A), INH-2 (C), INH-3(A), INH-1 (sd) and INH-3 (sd) formulations were 102.33 ± 1.19%, 102.33 ± 1.49%, 101.32 ± 1.83%, 102.39 ± 1.77%, 101.48 ± 1.18%, respectively. The results suggest that the overall process of mixing was accurate and able to produce uniform distribution of drug in the formulations. Uniform content was achieved by both the mixing and spray drying procedures.

3.5 Morphology of dry powder formulations

Scanning electron micrographs show that the particle shape of the milled drug, carriers and spray dried formulations varied from irregular shapes to spherical shapes (Fig. 1). Fig. 1A-1G are the micrographs of micronised drugs, micronised sugar carriers and fine sugar carriers ground by the grinding mill. Fine lactose is mainly in irregular shape. Trehalose and mannose particles appeared to be slightly more spherical than lactose. The mannose particles are more symmetrical and clearly rounder than either trehalose or lactose. In fact the micronised particles tend to be cohesive and form agglomerates as were apparent from the scanning electron micrographs. There was no distinguishable difference in the morphology of these micronised or fine particles.

The spray dried particles (Fig. 1H-1I) are typically spherical particles with 5-10 μm in size. Scanning electron micrographs show that the particle shape of spray dried formulations varied from spherical shape with a rough surface to spherical shape with smooth surface. Fig. 1H and 1I also show that a number of small particles adhering to a

large particle. This is similar to those of fine trehalose or fine lactose particles obtained from grinding mill (Fig. 1E and 1G).

3.6 In vitro deposition of isoniazid to lower stage of the ACI

Fig. 2 depicts the particle size distribution of a single dose delivery from selected isoniazid dry powder formulations [INH-1 (A), INH-2 (C), INH-3 (A), INH-1 (sd) and INH-3 (sd)]. Each bar represents the powder of certain size ranges collected on a defined stage of the ACI. These results show the weight fraction according to the size distribution of the aerosolized particles of isoniazid with different types and ratio of micronised and fine carriers in physical mixing formulations and spray dried formulations.

The ED of all formulations varied from 4.58 to 5.73 mg (nominal dose is 6 mg) when calculated from ACI (Table 2). Different delivery efficiency of a formulation based on aerodynamic diameters is expected. FPFs of selected formulations are shown in Table 2. These selected formulations had FPF over 60% and all formulations can travel to stages 4-7 of the ACI (Fig. 2) which is predicted to be the alveoli. Whereas large particles stayed at earlier stages (stage 0-2). GSDs of all formulations are 1.53 to 1.97, therefore these formulations are polydisperse which are typical for dry powder aerosols.

The average MMAD of each powder formulation is represented in Table 2. The MMAD varies from 3.14 to 5.85 μm which is ideal range for pharmaceutical aerosols. When the

initial MMAD of formulation was compared with the MMAD after 3 months storage, it was found that the formulation of isoniazid mixed with fine trehalose or micronised mannose showed MMAD values were significantly larger than the initial values ($P < 0.05$). The significant increase of MMAD after storage was an evidence of physical instability, which may cause from moisture sorption of the carrier. Whereas formulation containing fine lactose shows that the MMAD values after 3 months storage are not significantly different ($P > 0.05$) from the initial size. The results suggest that these formulations have high physical stability. In contrast, the spray dried isoniazid with lactose showed MMAD after 3 months storage lower than the initial value. It may be explained by the effect of dry heat on the formulation during initial evaluation. After storage for a period of time the heat dissipated resulting in more realistic size than the initial size. For spray dried isoniazid containing trehalose, the MMAD increased significantly (Table 3) after 3 month storage. This may cause particle agglomeration from moisture uptake.

3.7 Drug susceptibility testing

Few particles were detected in the M7H9 medium (Fig. 3 A) which did not interfere the histogram of event. Histogram profiles of the events in the following suspension are; unstained viable *M. bovis* cells (Fig. 3B), viable *M. bovis* cells stained with FDA (Fig. 3 C) and viable *M. bovis* cells incubated with isoniazid 0.3 μg/ml for 24 h and then stained with FDA (Fig. 3D). Only viable *M. bovis* organisms demonstrated a fluorescent intensity, with a mean channel fluorescence of 1,000 (range of 100-4,000). Unstained viable *M. bovis* cells were detected at a channel of 1-10 (Fig. 3B). Whereas non-viable

M. bovis had a channel of 10-100. When these experiments were repeated, similar results were obtained. From the results, it can be deduced that the channel of event correlated with viability or non-viability of mycobacteria organism which was in agreement with previous reports (Norden et al., 1995).

Figure 4 shows the number of viable *M bovis* cells after incubation with the selected formulations and standard isoniazid. All concentrations of isoniazid formulations killed more than 99.9% of the *M. bovis* population (originally $1^* 10^6$). The results show that the ability of these formulations to kill *M. bovis* cells were equal to the ability of standard isoniazid ($P < 0.05$) at all concentrations.

The MIC of the formulations and isoniazid standard on *M. tuberculosis* compared by broth microdilution method (Coban et al., 2004; Leite et al., 2000) was carried out. The results show that the MICs of these formulations were from 1.7 to 3.14 times lower than the standard isoniazid (Table 3). These results indicated that the formulations were more effective than pure isoniazid. The MICs against *M. tuberculosis* were close to formerly reported (Kirk et al., 1998a; Mohamad et al., 2004; Norden et al., 1998).

4. Conclusions

Physical mixing of isoniazid with micronised and fine trehalose, mannose or lactose and spray drying of isoniazid mixture with trehalose or lactose can be used to produce dry powder for use as aerosols. Dry powder formulations can be used for lung delivery of this antituberculosis drugs because they can travel to stage 4 and below although the percentage reaching stages 6 and 7 was quite small (Figure 2). Spray dried lactose

formulation maintained high physical and chemical stability over 3 months storage. All formulations have shown similar antimycobacterial activity against *M. bovis*. The MICs of isoniazid formulations against *M. tuberculosis* are 1.7 to 3.4 times lower than standard isoniazid. The reasons behind this improved activity against M. tuberculosis should be further investigated. Physical and chemical stability after longer storage period should also be investigated to determine the actual shelf-life of the isoniazid dry powder formulations.

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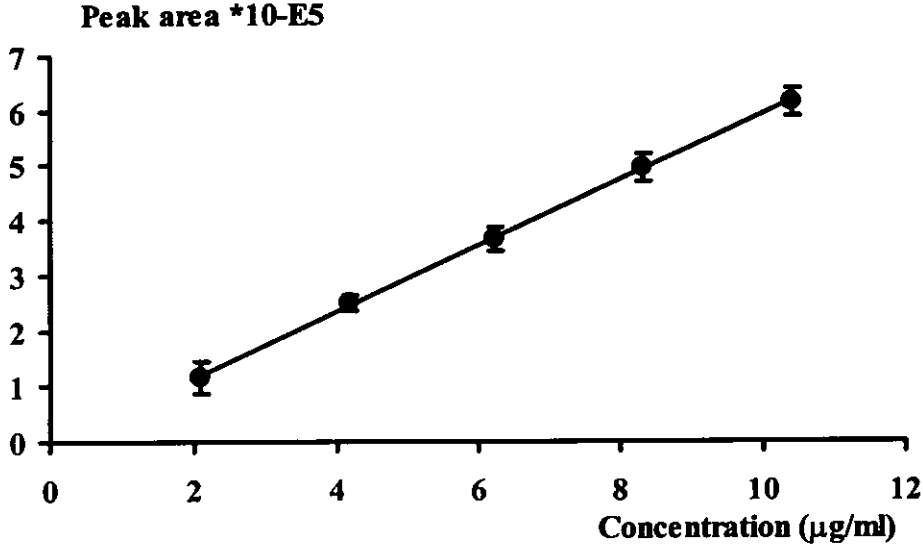

Fig. 1 Standard curve of isoniazid.

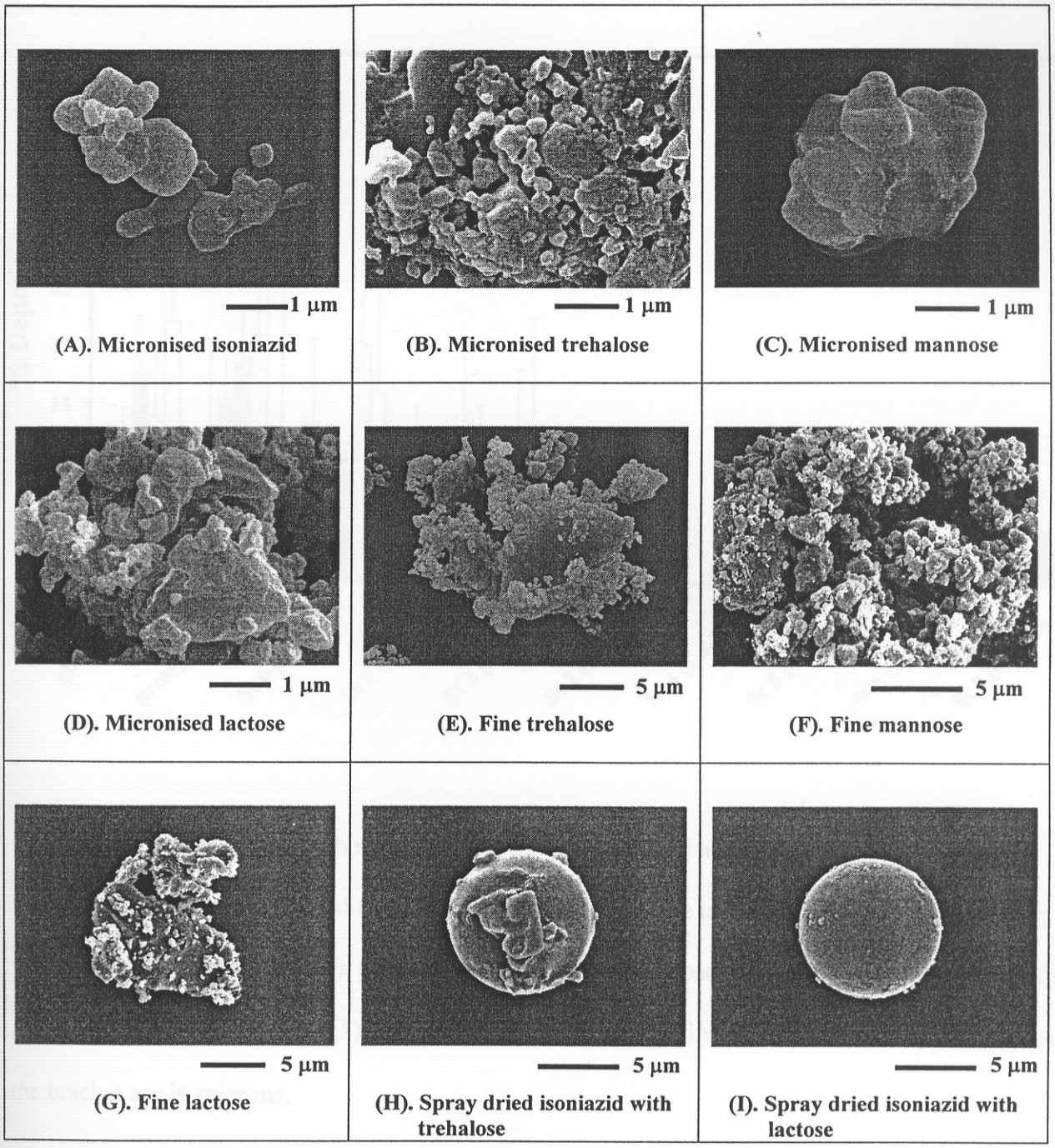

Fig. 2 Scanning electron micrographs of micronised isoniazid, micronised and fine carriers and spray dried formulations.

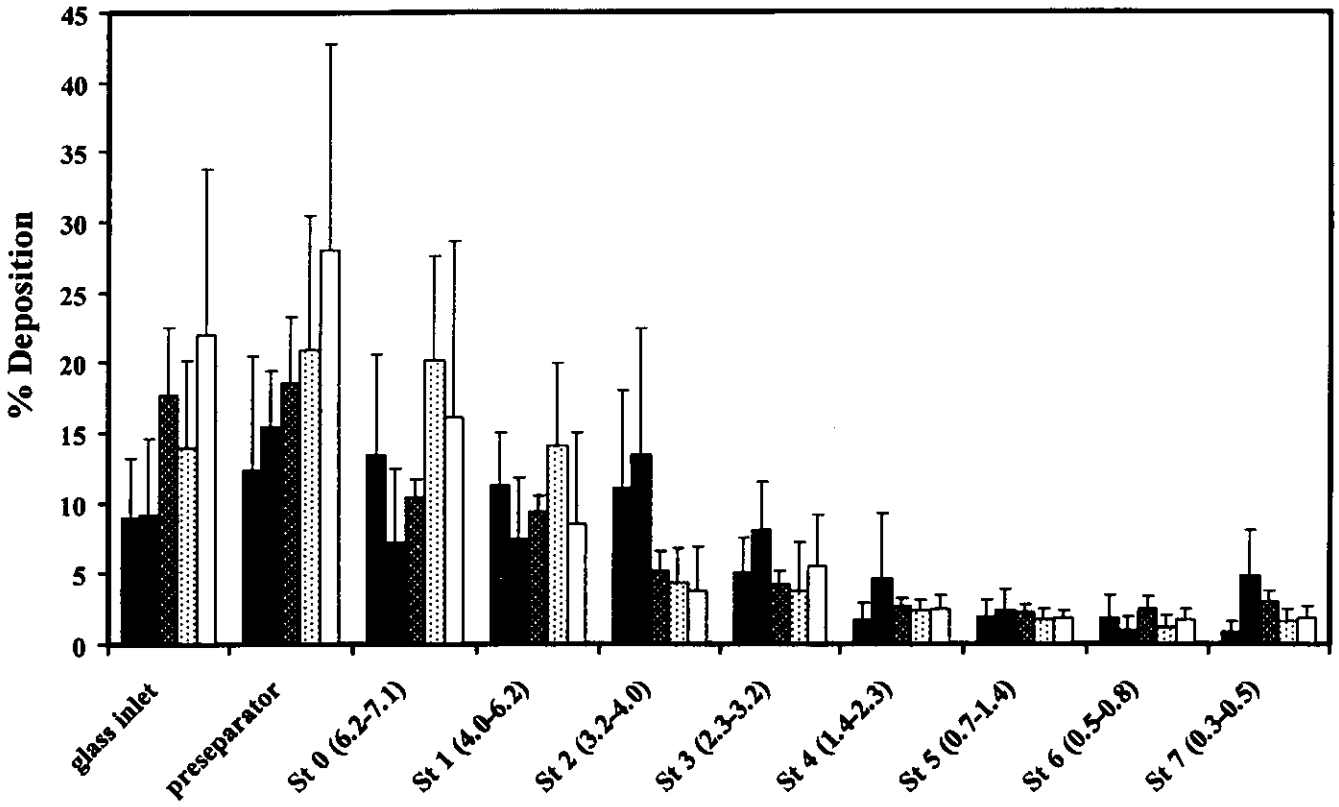

Fig. 3 Size distribution on each stage of the ACI as aerosolized at a flow rate of 60 l/min of isoniazid formulations obtained from physical mixing with fine trehalose (■), physical mixing with micronised mannose (■), physical mixing with fine lactose (▩) and isoniazid obtained from spray drying with trehalose (⊡), spray drying with lactose (□) (mean ± SD, n = 6). The values in the bracket are in microns.

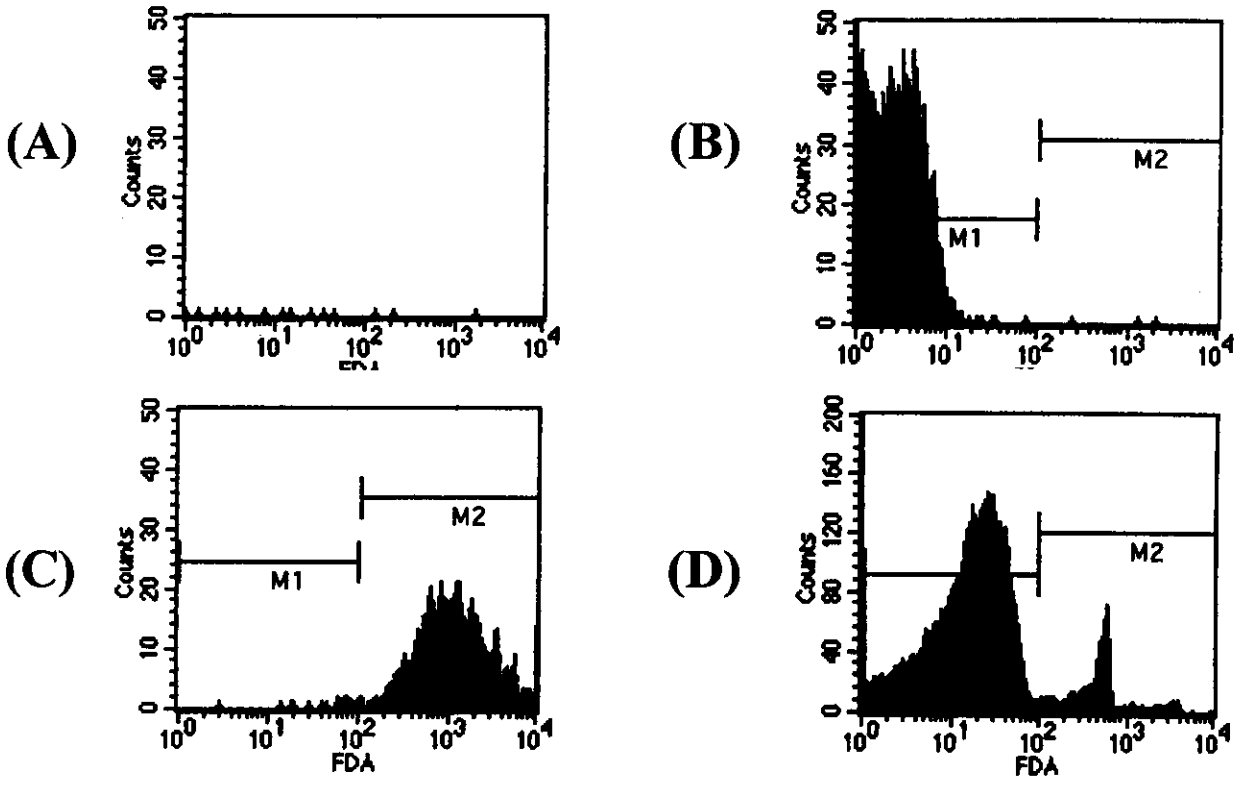

Fig. 4 Histogram profiles of the intensity of fluorescence based on the number of events; (A) M7H9 medium, (B) unstained viable *M. bovis* cells, (C) viable *M. bovis* cells stained with fluorescein diacetate, (D) viable *M. bovis* cells incubated with 0.3 μg/ml of isoniazid for 24 h and then stained with fluorescein diacetate.

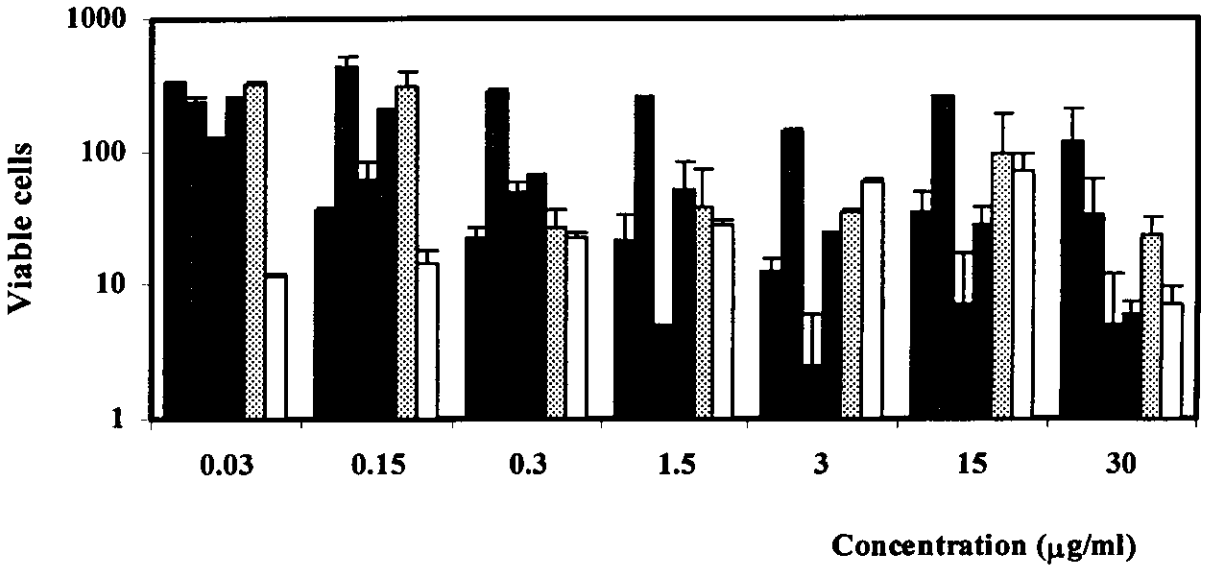

Fig. 5 The histograms showing the number of viable *M. bovis* cells after 24 h incubation with various concentrations of isoniazid solutions prepared from dry powder formulations, isoniazid alone (■), physical mixing with fine trehalose (■), physical mixing with micronised mannose (■), physical mixing with fine lactose (■), spray dried with trehalose (▩), spray dried with lactose (□).

Table 1

Compositions of dry powder formulations

Method	Formulation no.	Formulation code	Drug (0.6 g)	Carrier (1 g)
Physical mixing	1	INH-1 (A)	Micronised isoniazid	Fine trehalose
	2	INH-1 (B)	Micronised isoniazid	Fine trehalose : micronised trehalose (1:1)
	3	INH-1 (C)	Micronised isoniazid	Micronised trehalose
	4	INH-2 (A)	Micronised isoniazid	Fine mannose
	5	INH-2 (B)	Micronised isoniazid	Fine mannose : micronised mannose (1:1)
	6	INH-2 (C)	Micronised isoniazid	Micronised mannose
	7	INH-3 (A)	Micronised isoniazid	Fine lactose
	8	INH-3 (B)	Micronised isoniazid	Fine lactose : micronised lactose (1:1)
	9	INH-3 (C)	Micronised isoniazid	Micronised lactose
Spray drying	10	INH-1 (sd)	Isoniazid	Trehalose
	11	INH-2 (sd)	Isoniazid	Mannose
	12	INH-3 (sd)	Isoniazid	Lactose

Table 2 RSD values and % recovery of isoniazid analysis by HPLC.

Concentration (μg/ml)	%RSD		% recovery (accuracy)
	Intra-day	Inter-day	Intra-day
2	1.75	1.78	98.41
4	1.56	1.42	99.21
6	0.91	1.36	102.32
8	0.80	0.85	101.21
10	0.29	0.23	100.96

Table 3 Volume median diameter (VMD) of isoniazid, sugar carriers and spray dried formulations

Materials	VMD (mean ± SD, n =3) (μm)
Micronised isoniazid	4.07 ± 2.23
Micronised lactose	6.04 ± 0.48
Micronised mannose	3.75 ± 1.73
Micronised trehalose	4.84 ± 1.81
Fine lactose	17.88 ± 2.25
Fine mannose	16.91 ± 1.44
Fine trehalose	15.21 ± 1.42
Spray dried isoniazid and lactose	6.08 ± 0.81
Spray dried isoniazid and trehalose	6.48 ± 1.50

Table 4

Percentage of the isoniazid content, FPF and MMAD of physical mixing and spray dried formulations (mean ± SD, n=6 except for % content which n = 10)

Method	Drug	Carrier	%Content		ED	FPF	GSD	MMAD	
			Initial	After 3 months	(mg)	(%)		Initial	After 3 months
Physical mixing	isoniazid	fine trehalose	102.33±1.19	102.69±2.02	4.58±1.06	64.74±2.26	1.62±0.36	4.40±0.65	5.33±1.04[a]
		micronised mannose	102.33±1.49	98.09±3.14	5.02±0.50	73.86±8.62	1.74±0.42	3.14±0.38	5.49±0.55[a]
		fine lactose	101.32±1.83	100.62±2.35	5.73±0.41	81.50±4.66	1.97±0.11	4.01±0.45	4.89±0.26
Spray drying	isoniazid	Trehalose	102.39±1.77	103.35±0.95	5.89±0.65	67.53±2.63	1.53±0.21	5.85±1.83	7.87±4.17
		Lactose	101.48±1.18	103.10±3.58	5.43±0.55	71.11±0.78	1.73±0.28	4.83±2.31	2.12±0.22[b]

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

Table 5
MICs of isoniazid and various isoniazid dry powder formulations against *M. tuberculosis* by broth microdilution method

Formulation code	MIC (μg/ml)
Isoniazid standard	0.1
INH-1 (A)	0.0293
INH-2 (C)	0.0586
INH-3 (A)	0.0293
INH-1 (sd)	0.0586
INH-3 (sd)	0.0586