Eur J Pharm Sci. 2008 Aug 7; 34(4-5):351

A Hand-Held Apparatus for "Nose-Only" Exposure of Mice to Inhalable Microparticles as a Dry Powder Inhalation Targeting Lung and Airway Macrophages

Jatinder Kaur[a], Pavan Muttil[a,b], Kaushlendra Kumar[a], Rahul Verma[a], Awadh Bihari Yadav[a], Rolee Sharma[a,c] and Amit Misra[a]*

[a]Pharmaceutics Division, Central Drug Research Institute, Lucknow, India

[b]Present Address: Division of Molecular Pharmaceutics, University of North Carolina School of Pharmacy, Chapel Hill, NC, USA.

[c]Present Address: Department of Microbiology, Integral University, Kursi Road, Lucknow, India.

*Correspondence: Amit Misra, Pharmaceutics Division, Central Drug Research Institute, Lucknow 226001, India. Phone: 91-522-261-2411 Ext 4302. Fax: 91-522-262-3405 Email: amit.cdri@gmail.com

Abstract

A hand-held apparatus designed for the purpose of administering dry powder inhalations to rodents by “Nose-only” exposure was standardized. An aerosol of microparticles containing the anti-tuberculosis drugs isoniazid and rifabutin was generated and the dose available for inhalation by rodents was determined by collecting microparticles emitted at the delivery port. Separate groups of mice received inhalations of microparticles, free rifabutin orally, or free rifabutin intravenously. Rifabutin was estimated in serum and tissues by HPLC. When ~20 mg of microparticles were loaded in the apparatus, ~2.5 mg were collected at the delivery port in 30 seconds of operation. Mice inhaled ~300 µg of the 2.5 mg emitted at the delivery port. Alveolar macrophages of mice receiving inhalations for 30 sec accumulated 0.38 µg of rifabutin, while the amount in blood serum of these mice was 0.62 µg. In mice receiving 83 µg rifabutin intravenously or orally, the intracellular amounts were 0.06 and 0.07 µg respectively, while the amounts in serum were 1.02 and 0.80 µg. These observations confirmed that inhalation of microparticles targeted lung macrophages. The dose available for inhalation was consistent with the duration of exposure (10.5-13.5 CV%) and its dependence on the amount of microparticles loaded (r^2=0.982), and on duration of exposure (r^2=0.992), was satisfactory.

Keywords: Dry powder inhalation; respirable microspheres; pulmonary delivery; tuberculosis; aerosol generation; inhalation dosimetry

Introduction

Pulmonary drug delivery by dry powder inhalation (DPI) is an active field of pharmaceutical research and development, but equipment for studies in rodents is not readily available. The related field of inhalation toxicology addresses inhalation of pollutants and hazardous substances, typically over long periods or during repeated exposure. Recent reviews of inhalation devices and experimental designs in pulmonary delivery of liquids and particles to rats indicate that there is a need for apparatus capable of short-term, nose-only administration of dry powders to rodents [1, 2].

The PennCentury Dry Powder Insufflator™ is a popular, commercially available, hand-held device for use with rodents. It, however, requires insertion of a tube into the trachea of the test animal, and the use of positive pressure to introduce the material into the lungs [1]. Other equipment designs for use in rodent studies on DPI either require tracheal intubation of the test animal [3-6], or "whole-body" exposure [7]. Although Ben-Jebira *et al.* (2000) reported pulmonary delivery to rodents by spontaneous respiration, methodologies involving insufflation generally use positive pressure to achieve pulmonary delivery. It is known that biodistribution of particles inhaled under ambient pressure through inspiration alone is different from that following inhalation under positive pressure or intra-tracheal instillation [8, 9]. Intra-tracheal instillation is an alternative to insufflation, but results in bypassing the nasopharynx, where a large proportion of inhaled particles is deposited during physiological breathing.

In the case of whole-body exposure, the substance meant for inhalation can enter the body of the test animal through orifices other than the nasopharynx or through percutaneous absorption. Some material is also retained on the fur of the animal. These possibilities

complicate dosimetry. However, portable whole-body exposure chambers are popular and are available for experimental use [10].

"Nose-only" exposure of drugs and toxicants is more favoured, since rodents are obligate nose-breathers [11]. "Nose-only" methods of inhalation by the test animal have been standardised and validated by several research groups, with understandable emphasis on close (and often computer-aided) monitoring of operating parameters such as air flow rate, air pressure, aerosol concentration, etc. [12, 13].

Apparatus designed for long-term inhalation exposure during inhalation toxicolgy studies must be capable of controlling aerosol characteristics and uniformity of inhalation dose delivery. For pulmonary drug delivery, however, the period of exposure required is of the order of seconds to minutes, so that maintenance of aerosol characteristics over prolonged periods is not crucial [14]. This report describes a simple apparatus to address the need of administering inhalable microparticles at ambient-pressure, through "nose-only" (but not flow-past) exposure, to mice.

Earlier reports from our laboratory have described an extremely simple device fabricated using a plastic centrifuge tube for "nose-only" administration of particulate aerosols to mice and rats under ambient pressure, without endotracheal intubation or tracheotomy [15-17]. The device is easy to fabricate and use. Earlier versions of our inhalation apparatus employed exhaust air from a diaphragm pump operated at very low speed to fluidize the particle bed [15]. Further work indicated that a pipette bulb may be used to manually generate the aerosol [16, 17]. This report therefore describes the standardization and validation of a hand-operated apparatus for administration of DPI to mice. The DPI used in these studies has been described in greater detail elsewhere [16]. It is anticipated that the demonstration of the

reproducibility of the inhalation apparatus will encourage other investigators to take up studies that might have seemed difficult due to lack of inhalation equipment.

Materials and Methods

Inhalable Microparticles

Microparticles containing isoniazid and rifabutin were prepared by spray-drying. Briefly, a true solution comprising one part each of isoniazid and rifabutin and three parts *poly*(lactic acid) w/v was spray-dried using a Buchi 190 spray-dryer. The solvent system was methanol and dichloromethane in a ratio of 3:22 v/v. The operating conditions were as follows: pump speed- 5 ml/min; air flow rate- 700–800 NL/h, inlet temperature- 55 ± 1 °C and outlet temperature- 32 ± 1 °C. Drugs were incorporated at efficiencies > 90% and the content of each drug was ~20% w/w. The size distribution of microparticles thus obtained was between 1 and 10 μm (median 4.3 μm) in suspension. A Mercer (Lovelace) cascade impactor and inhalation equipment from In-Tox Products (Albuquerque, NM, USA) was used to determine mass median aerodynamic diameter (MMAD). The MMAD was 3.6 μm, and the fine particle fraction (FPF) of the powder was 68% when estimated as described elsewhere [16].

Fabrication and Operation of the Apparatus

The components of the device included: (1) A tapered *poly*(propylene) centrifuge tube of capacity 15 ml (Greiner) that formed the powder fluidization or aerosol generation chamber; (2) Flexible, C-Flex tubing (Sigma, T8288-25FT) of ~1.5 × 0.8 × 3 mm (internal diameter. × wall thickness × outer diameter) to introduce a turbulent fluidizing air stream into the chamber; and, (3) a rubber pipette bulb that provided the source of turbulent air when pressed and released. Two holes were bored in the centrifuge tube. The first hole was made in the apex of the taper to admit the flexible tubing. The second hole was made in the wall of the

tube, near the rim of the screw cap, to serve as the Delivery Port for nose-only inhalation. The apparatus was designed for use with only one animal at a time (Fig.1).

Centrifuge tubes are routinely used in cell culture labs and mass-produced by a large number of laboratory plasticware suppliers. These tubes have a tapered bottom and a screw cap at the top. Their internal geometry is similar to equipment generally employed for powder fluidization by a turbulent fluidization flow regime [18]. The apparatus reported here was designed to fluidize a powder bed within the confines of the centrifuge tube by means of a turbulent air stream.

The fabrication and operation of the apparatus is graphically represented in Figure 1. The device was fabricated as follows. A 15 ml centrifuge tube was placed cap-down on the workbench. Thus inverted, the screw cap represented the 'base' while the taper formed the 'top.' Aluminum wire of ~3 mm diameter was heated on a flame and inserted in the apex of the taper to make a hole indicated as "Hole for Air Inlet Tube" in Figure 1B. The hot wire was twisted to enlarge the hole in the apex, till it was large enough to admit the flexible tubing, leaving annular space of about 1 mm between the tubing and the rim of the hole. About 20 cm of C-Flex tubing was then taken, and one end inserted into this hole to a distance of ~2.5 cm from the 'base'. The other end of the flexible tubing was connected to a rubber pipette bulb. A graduation mark showing a volume of 12 ml on the wall of the centrifuge tube was convenient for locating the open end of the aeration tube. When the aeration tube was level with the 12 ml mark, the fluidizing air stream entered the apparatus from the same point during every use. Annular space between the wall of the centrifuge tube and the aeration tube prevented the build-up of internal pressure in the centrifuge tube.

The delivery port was made at a height of ~1.5 cm from the base (Figure 1B) using a hot glass rod. The diameter of this hole was ~25 mm, and its center was located at the graduation mark indicating 13.5 ml on the wall of the centrifuge tube. Care was taken to leave a smooth edge to avoid discomfort to the animal.

Pre-weighed microparticles were placed in the cap of the tube. A test animal was then restrained with its nares inserted in the delivery port, without touching the powder bed. The pipette bulb was pressed and released once every second over the desired period of exposure, to fluidize the powder bed and create a 'dusty' atmosphere for the animal to breathe in.

Dose Available for Inhalation

Different amounts of microparticles (10 to 50 mg) were taken for fluidization. The amount taken for fluidization is henceforth referred to as the 'charge.' Microparticles were fluidized for 30, 60 or 90 seconds. All experiments were conducted in quadruplicate. Pre-weighed wads of cotton wool (~200 mg) were used to completely occlude the delivery port during operation. The surface of the wad exposed to the fluidized powder remained flush with the inner wall of the tube. The amount of microparticles collected on the plug was first determined by weighing on a 5-digit analytical balance (Mettler AE163). In addition, rifabutin was extracted from the microparticles by repeated vortexing with 5 × 5 ml portions of methanol and assayed by HPLC as described below [16]. The amount of microparticles collected on the plug was then calculated by multiplying the amount of rifabutin found by four.

HPLC Assay of Rifabutin in Microparticles and Cotton Wool

The analytical method reported by Lewis [19] was used with minor modifications. The HPLC system consisted of a LC-10ADVP pump and an SPD-10AVP UV detector

(Shimadzu) set at a wavelength of 275 nm. Chromatographic separations were performed on a Phenomenex C-18 (250 x 4.6 mm, 5 μm) Luna column, coupled with a C18 guard column. The mobile phase was composed of acetonitrile: phosphate buffer:: 55:45% v/v, pH 4.10. Rifabutin eluted at 6.3 min at a flow rate of 1 ml/min. Data was analyzed using Shimadzu CLASS-VP software (Version 6.2).

A standard curve was constructed using rifabutin Analytical Standard (AS) dissolved in methanol. Corresponding amounts of microparticles were weighed and extracted with methanol. In parallel, microparticles were completely dissolved in dichloromethane and the polymer re-precipitated by adding methanol. An aliquot of the supernatant was evaporated to dryness, reconstituted in methanol and analyzed as described above. No significant difference (one-way ANOVA, $P<0.001$) was observed between results of the two extraction methods (data not shown).

In Vivo *Administration and Sampling*

All animal experiments were conducted after obtaining approval from the Institutional Animal Ethics Committee of the Central Drug Research Institute. Groups of four male Swiss mice weighing 24 ± 1 g were formed. Each mouse in the first group received intravenous injections of 83 μg rifabutin dissolved in 100 μl. The solvent system used to prepare this solution comprised three volumes of ethanol and seven volumes of phosphate buffered saline at pH 5.1. Rifabutin was initially dissolved in ethanol, and the buffer added to make up volume. This solution was sterile-filtered through a 0.22 μm membrane prior to injection.

The second group of animals was administered the same solution, without sterile filtering, by oral gavage. The third and fourth groups received inhalations, with 20 mg microparticles

charged into the apparatus, and exposure times of 30 or 60 sec. Two additional mice provided untreated control samples.

After dosing, animals were anesthetized by intraperitoneal administration of 60 mg/kg ketamine and 6 mg/kg xylazine [20] and their thoracic cavity opened. Cardiac puncture was carried out for exsanguination. Blood serum was separated by centrifugation for 5 min at 10,000×*g* and stored at -20°C till analysis. Bronchioalveolar lavage was conducted to recover airway and alveolar macrophages as described earlier [15]. Briefly, the trachea was canulated and lungs were lavaged four times with 1.5 ml of chilled saline. All lavages were pooled and centrifuged at 3,000×*g* to recover airway and lung macrophages. The cell yield from each mouse was determined using a haemocytometer. The supernatant and cell pellet were separately assayed by HPLC for rifabutin content. The lungs, liver and kidneys were also collected. Sampling was conducted within 5-10 minutes of intravenous administration or inhalation, but 2 hrs (~t_{max}) were allowed to elapse after oral administration for ease of comparison with published data [21].

HPLC Analysis of Biological Samples

HPLC conditions were the same as above. Calibration standards for assay of rifabutin in cells or tissues were prepared in lysates of cultured THP-1 monocytes. Normal mouse serum was used for preparing calibration standards for analysis in serum. Cells were counted and lysed by ultrasonication using a probe sonicator (VibraCell). A lysate of 0.5×10^6 cells or 500 µl of serum was spiked with appropriate volumes of rifabutin and *poly*(lactic acid) stock solutions, keeping the volume of organic phase ≤/5 % of the biomatrix. Concentrations spanning nine points between 0.1 and 7 µg/ml of rifabutin, plus corresponding amounts of polymer

expected in microparticles were thus prepared. Quality control samples at low (0.5 µg/ml), medium (4 µg/ml) and high (10 µg/ ml) concentrations were used for partial validation.

Rifabutin was extracted from standards and samples using Lewis' procedure with minor modifications [19]. Briefly, aliquots of blank, spiked or test samples (500 µl) were taken in a 10 ml graduated glass tube and extracted with 0.5 ml of a mixture of n-hexane-ethyl/acetate (80:20,v/v), vortex-mixed and centrifuged (Biofuge Stratos, Kendro) for 5 min at 3000×*g*. The top organic layer was decanted into glass tubes after snap-freezing the lower aqueous layer in liquid nitrogen. This process was repeated two more times. The combined organic fraction was evaporated to dryness in a vacuum concentrator (Maxi-dry Lyo, Heto-Holten, Denmark). The dry residue was reconstituted in 100 µl mobile phase, vortex-mixed and centrifuged, and the clear supernatant (50 µl) was injected onto the HPLC column.

The method of extraction used with cell lysates proved inappropriate for extraction from tissue due to spillover of large amounts of lipids. An alternative extraction method developed by Calleja *et al* [22] for rifampicin was therefore adopted with modifications. The fresh (wet) weight of tissue samples was recorded and the tissue homogenized at ~20,000 rpm in 10 ml phosphate-buffered saline (Ultra-Turrax, Ika Werke, Germany). The homogenate was stored at -20°C till analysis. Prior to analysis, samples were thawed and centrifuged at 10,000×*g* for 10 min. The supernatant was decanted and about 100 mg of the pellet was weighed out in a glass tube. The tissue sample was vortexed for 30 sec in triple distilled water to lyse cells. A solution of 0.02% w/v butylated hydroxy toluene (BHT) in 200 µl acetonitrile was added to stabilise rifabutin against oxidation [23] and vortexed for another 30 sec. Rifabutin was extracted by vortexing with three sequential portions of 1 ml each, composed of equal parts of dichloromethane and *n*-hexane and containing 0.02% w/v of BHT. The extracts were

pooled, evaporated to dryness and the residue reconstituted in 100 µl acetonitrile containing 0.02% w/v BHT. This was injected on to the column. The supernatant of the tissue homogenate was processed identically.

Curve Fitting

Saturability of particle transport to the cotton plugs during dose determination was assessed by fitting a Boltzmann function to the data obtained in standardization experiments. The ratio of the dose available for inhalation to the charge placed in the apparatus (the dependent variable) plotted on the y axis was fitted to the trial number on the x axis [24]. Goodness of fit represented by the χ^2 value indicated whether the curve was sigmoidal in nature when the equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2$$

was employed. Here, A_1 and A_2 refer to the initial and final values of dose:charge ratio, respectively and x_0 refers to the inflection point at which the function attained half-height. The differential *dx* defines the rate constant.

Polynomials of the first order were fit to standard curve data by least-squares regression, while a second-order polynomial was similarly fitted to data on the dependence of dose available for inhalation on duration of operation.

Results

Analytical Methods

The analytical method permitted baseline separation of rifabutin from extractives spilling over from cotton wool as well as extracts of the three biomatrices (cell lysate, serum and tissue) tested. A chromatogram showing the rifabutin peak in a serum sample is illustrated in

Fig 2A. Linear calibration curves were generated with analytical standards (Fig. 2B) and working standards (Fig. 2C) for quantitation in cotton wool. Regression equations fitted to standard curve data showed regression coefficients (r^2) > 0.995. Corresponding curves for bioanalysis are depicted in Panels D and E of Fig. 2.

Rifabutin recovery was 90.3 ± 1.86, 96.3 ± 1.32, 94.5 ± 2.89 % respectively from low, medium and high concentration quality control samples prepared with spiked cotton wool (nine injections per concentration). In case of biomatrices, these values were 83.6 ± 2.16, 87.6 ± 3.57 and 84.7 ± 2.09 % respectively (n=6).

"Priming" is Required for Dose Uniformity

A freshly fabricated apparatus required priming before constant doses could be obtained. About 10 mg microparticles were accurately weighed into the cap of a freshly fabricated apparatus and the pipette bulb actuated 30 times. A with a wad of cotton wool covered the delivery port during operation. The process was repeated ten times with the same apparatus. Steady increments in the dose available for inhalation were observed in the first three attempts, after which a plateau was reached. Uniformity was satisfactory from the fifth attempt onwards, most probably due to 'priming' or coating of the internal walls with the powder, as encountered even in marketed devices intended for human use [25]. The walls of the tube accumulated a coating of microparticles during the first few operations, as revealed by the characteristic colour of rifabutin. This priming was saturable, and once accomplished, stabilised the dose available for inhalation at the delivery port. Fig. 3A shows the stabilisation graphically. Fitting the Boltzman equation to the ratios, without weighting, resulted in values of 0.019869 and 0.10727 for initial (A1) and final (A2) coefficients. The

function attained half-height (x_0) at trial number 2.4966. Goodness of fit was adequate, with a χ^2 value of 0.5 $\times 10^{-5}$ and r^2 of 0.965.

Multiple Doses from a Single Charge

The performance of the apparatus in terms of multiple dose delivery from a single charge was evaluated with a constant amount of microparticles placed in the cap. For a fixed charge (20 mg) and duration of operation (30 sec), consistent amounts were not collected on the plug after the second or third dose as shown in 3B. The trend of microparticle transport following aerosolization was explored by fitting a Boltzmann function to the data as above, in order to check whether the values were maintained during multiple operations. Weighting was provided by the standard deviations from the means of four datasets. For this fit, χ^2 was close to 0.5, whereas r^2 was 0.998.

Dose Available for Inhalation Depends on Charge and Duration of Exposure

Figure 3C shows the dose available for inhalation when the charge in a primed apparatus was varied from 10.5±0.32 to 20.72±0.27, 30.15±0.10, 40.35±0.19 and 50.4±0.18 mg. The duration of exposure was kept fixed at 30 seconds. Weighing the wads of cotton wool yielded sufficiently consistent results, but HPLC analysis revealed that these were underestimates. The concordance between gravimetry and HPLC estimation was greater at higher values of charge. When the charge was 10 mg, the concordance was 83.95±3.18%, rising to 93.88±0.29% at a charge of 50 mg. Only minor differences were observed in dose delivery when the charged amount was 10, 20 or 30 mg. When the charge was increased to 40 and 50 mg, the dose available for inhalation showed an asymptotic increase.

Figure 3D indicates the relationship between the duration of exposure and the dose available for inhalation. At a charge of 20.73±0.23 mg, 30 actuations over 30 sec (once every second)

led to the collection of 2.46 ± 0.26 mg as dose available for inhalation when determined by HPLC (2.1 ± 0.26 by gravimetry; concordance >85%).

Drug Amounts In Vivo

Animals received inhalations with 20 mg charge over 30 sec. A partial material balance was established by estimating the amounts of rifabutin in serum and tissues of the treated mice (Fig. 4). About half the amount of rifabutin administered intravenously or orally could be traced by analysing the serum and tissue samples. Thus, out of 83 μg administered to each mouse by intravenous injection or oral gavage, 40 and 38 μg respectively were found in the sampled compartments. Similarly, after microparticles were inhaled for 30 or 60 sec, 15 or 16 μg of rifabutin could be recovered. Comparing serum levels of rifabutin between groups by one-way ANOVA, there were no significant differences between mice receiving intravenous and oral doses at $P<0.01$. When either of these groups was compared with the groups receiving inhalations, significant differences were evident at $P<0.0001$. Rifabutin found in tissues following oral or intravenous administration showed the trend: liver>lungs>spleen, in agreement with the results of Battaglia *et al*. [21]. Drug amounts estimated in airway and lung macrophages recovered from animals receiving inhalations were >12 times the amounts in macrophages of intravenously- or orally-dosed mice.

Discussion

Aerosol generation is extremely important for efficient administration of inhalations. With special reference to DPI, pneumatic aerosolisation of a powder bed is usually achieved in "dust generators" that use either carrier beads or high-pressure (10-15 psig) to de-aggregate and fluidize individual particles [14, 18]. Dust-generating equipment is also designed to create a stable aerosol within a short span of time, and maintain aerosol characteristics over

several hours of operation. While the objectives of inhalation toxicology are best addressed by equipment capable of long-term, stable operation, pulmonary drug delivery does not necessarily require such equipment. Apparatus that can emit reproducible doses within the short period of a few seconds, and exhibits reasonable control on the dose available for inhalation, should serve the purpose of pharmaceutical aerosol inhalation.

The apparatus design reported here is simple, but serves its purpose satisfactorily. Provided that the powder used is suitable for use as a DPI, a centrifuge tube bearing two holes can be used to fluidize it, and administer reproducible, quantifiable doses to rodents. Further, each individual apparatus can be calibrated, either by weighing the doses collected on an inert matrix placed over the delivery port, or using more sensitive analytical techniques such as HPLC. Relatively few precautions ensure reproducible performance. The placement of the aeration tubing at a constant height above the powder bed was ensured by visual confirmation of coincidence of the end of the aeration tubing with the 12-ml graduation mark on the wall of the centrifuge tube. The necessity of "priming" the apparatus with a few blank runs became apparent from visual inspection of cotton wads held against the delivery port.

Once the apparatus was primed, the dose available for inhalation was proportional to the amount taken for fluidization (the charge) as well as the duration of exposure (Figure 3). The ratio of the dose available for inhalation to the charge proved a convenient metric to normalise differences in amounts of powder weighed for each operation. This metric may also be used for periodic validation of the apparatus. In our hands, no significant change in the dose: charge ratio was observed when the apparatus was tested during the 25th and 50th operation (data not shown).

Limitations

The limitations of the apparatus reported here can be listed as follows. First, it is not suitable for pulmonary delivery of powders that require highly turbulent airstreams for de-agglomeration of the DPI [26]. The fact that it performs satisfactorily with a powder having a large fine particle fraction [16] does not guarantee that it will work for powders that have a lower proportion of fines. Second, this version of the apparatus is not suitable for multiple dose delivery at a charge of 10 or 20 mg. The apparent consistency between the first four doses (Fig. 4) was misleading, since microscopic examination of the powder recovered on wads of cotton wool after each operation revealed appreciable differences in particle size distribution (data not shown). Whereas the amounts collected on the cotton wool plug were similar in terms of the content of 'fines' (<3 μm) for the first and second operation, subsequent trials showed progressive accumulation of larger particles. Other researchers may arrive at different conclusions, especially if the powder used is superior in aerosol characteristics and larger amounts are charged into the reservoir. Third, there is a lack of linearity in its performance. When the dose available for inhalation over duration of 30 sec is plotted against the charge, the curve describing their relationship rises asymptotically (Fig. 3C). Similarly, keeping the charge fixed at 20 mg and varying the duration of exposure results in a parabolic rather than linear increase in the dose available for inhalation. These limitations have implications regarding the nature of powders that can be used, the absolute amounts of powders that would be required to for charging the device, and the need for standardizing the duration of exposure with reference to charge through several experiments. Finally, since such apparatus is manually fabricated, each unit requires individual calibration.

Despite these limitations, the apparatus holds promise for experimenters constrained by the availability of equipment for "nose-only" exposure at ambient pressure in rodent studies.

Individually weighing ~20 mg microparticles as charge for each operation resulted in inhalation of similar amounts by mice over exposure times of 30 as well as 60 sec (Fig. 4). This result suggests that the animals were exposed to saturating inhalation doses under the two conditions, since the dose available for inhalation increased by about 20-25% when the duration of exposure was doubled to 60 sec (Fig. 3D).

Inhalation Dosimetry

Data in Figure 4 compared well with the exhaustive delineation of rifabutin biodistribution and pharmacokinetics carried out by Battaglia *et al*. [21]. These investigators administered ^{14}C-labeled rifabutin to rats and estimated drug amounts in 13 different tissues and the whole carcass to arrive at a material balance. They also calculated ratios of tissue concentrations to plasma concentrations at various time-points after oral administration. For instance, 2 h after a single oral administration, the ratio obtained by dividing the liver concentrations with the corresponding plasma concentrations was 28.86. In the case of the lungs, the ratio of tissue to serum concentration was 20.92. Thus, tissue concentrations may be calculated within a reasonable margin of error if the serum/plasma concentration is known. This approach was adopted to project a more complete material balance, using serum concentration data plotted in Figure 4. Corrections were applied by including the amounts recovered in bronchio-alveolar lavage fluid and lung macrophages shown in the same Figure. The results are shown in Table 1, which, for brevity, lists only the group means.

Since Battaglia *et al.* sampled tissue compartments 2 h after a single oral dose, mice administered rifabutin by oral gavage were used to benchmark the accuracy of results obtained during the present investigations. As evident from Table 1, the material balance accounts for 80.29 μg (96.39%) of the oral dose. Amounts directly determined in the liver,

lungs and spleen from mice receiving oral doses were within 79-83% of the projected amounts. In case of immediate sampling of tissues after intravenous injection, the errors were larger (62-73%), most likely due to differences in biodistribution and elimination parameters distinguishing the two routes. Despite lower amounts being detected by direct determination, the projected inhaled dose was 102 µg, representing an overestimate by 18.8% of the 83 µg actually administered.

The acceptable concordance [27] between results of direct determinations and the projected amounts after oral administration represents a valid picture of the biodistribution of rifabutin after oral administration. Delivery by inhalation, however, is more likely to approximate intravenous injection, except for the propensity of inhalation delivery to display a "pharmacokinetic capacitance" that down-modulates the blood level peak observed with vascular administration [28]. Even with the caveat that blood: tissue ratios might be further distorted by such capacitance on pulmonary delivery; the projected amounts of total doses inhaled appear plausible. Thus, on the basis of the projected rifabutin material balance, mice inhaled 61.5 and 66.5 µg of the drug in 30 and 60 sec, respectively. In terms of microparticle equivalents, these amounts may be stated as 307.7±6.5 and 332.7±8.2 µg, respectively.

Microparticles used here contain unusually large drug payloads, and therefore display significant "burst release" [16]. The drugs released in the initial burst on the pulmonary epithelium may be expected to distribute rapidly in various tissue compartments. In direct determinations, appreciable amounts of rifabutin were detected in serum, liver lungs and spleen. These followed the same trend in biodistribution as documented by Battaglia *et al* [21] when the route of administration was intravenous or oral. Inhalation delivery resulted in disrupting this trend. Larger amounts of rifabutin were found in the lungs rather than the liver

in animals receiving inhalations. Further, comparing cytosolic concentrations in lung macrophages with serum concentrations, it was evident that inhalations led to significant drug targeting. Whereas ratios of intracellular: serum amounts were 0.06 and 0.09 in the case of intravenous and oral administration, these ratios were 0.61 and 0.73 when inhalations were administered for 30 or 60 sec respectively.

Conclusions

An extremely simple single-dose apparatus was fabricated and calibrated for administration of DPI to mice. The inhaled dose of microparticles containing anti-TB drugs was determined and found to be ~15% of the dose available for inhalation at the delivery port. In contrast to intravenous or oral administration, inhalation targeted larger proportions of the rifabutin to the lungs in comparison to the liver. Targeting to alveolar macrophages was established by comparing relative amounts in these cells after intravenous, oral and inhalation dosing. The apparatus may help investigators to optimally utilize their grants for research on administration of DPI to mice.

Acknowledgments

PM, ABY and RS received Senior Research Fellowships of the Council of Scientific and Industrial Research, India (CSIR) during the course of this work, which was funded by the CSIR's NMITLI Program. This is CDRI Communication Number 7098.

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Figures

Figure 1

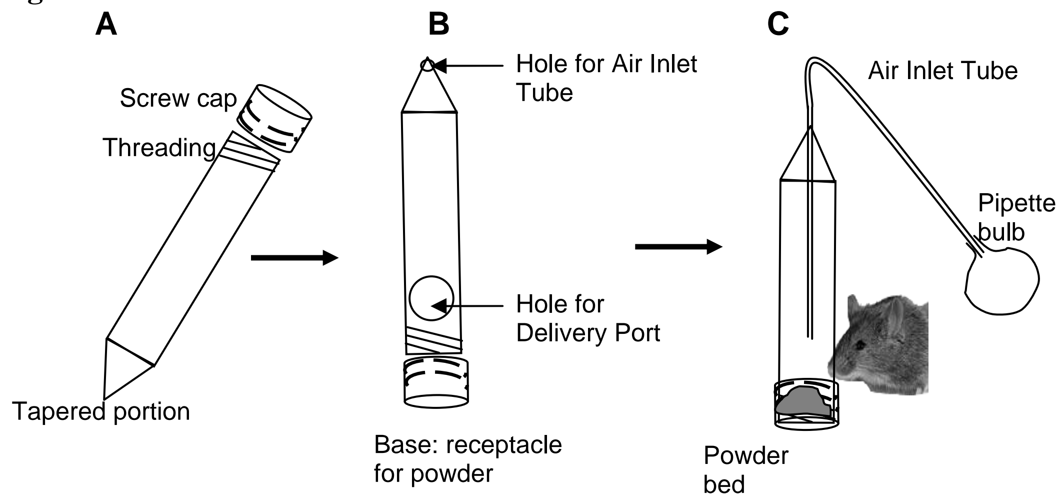

Figure 1: Fabrication of inhalation apparatus from a plastic centrifuge tube used in cell culture. A hole was bored in the apex of the taper to admit the air inlet tube. Another hole capable of accomodating the muzzle of a mouse was made in the wall of the tube to serve as the Delivery Port. A pipette bulb connected to flexible tubing was actuated to admit a turbulent airstream for fluidizing the powder.

Figure 2

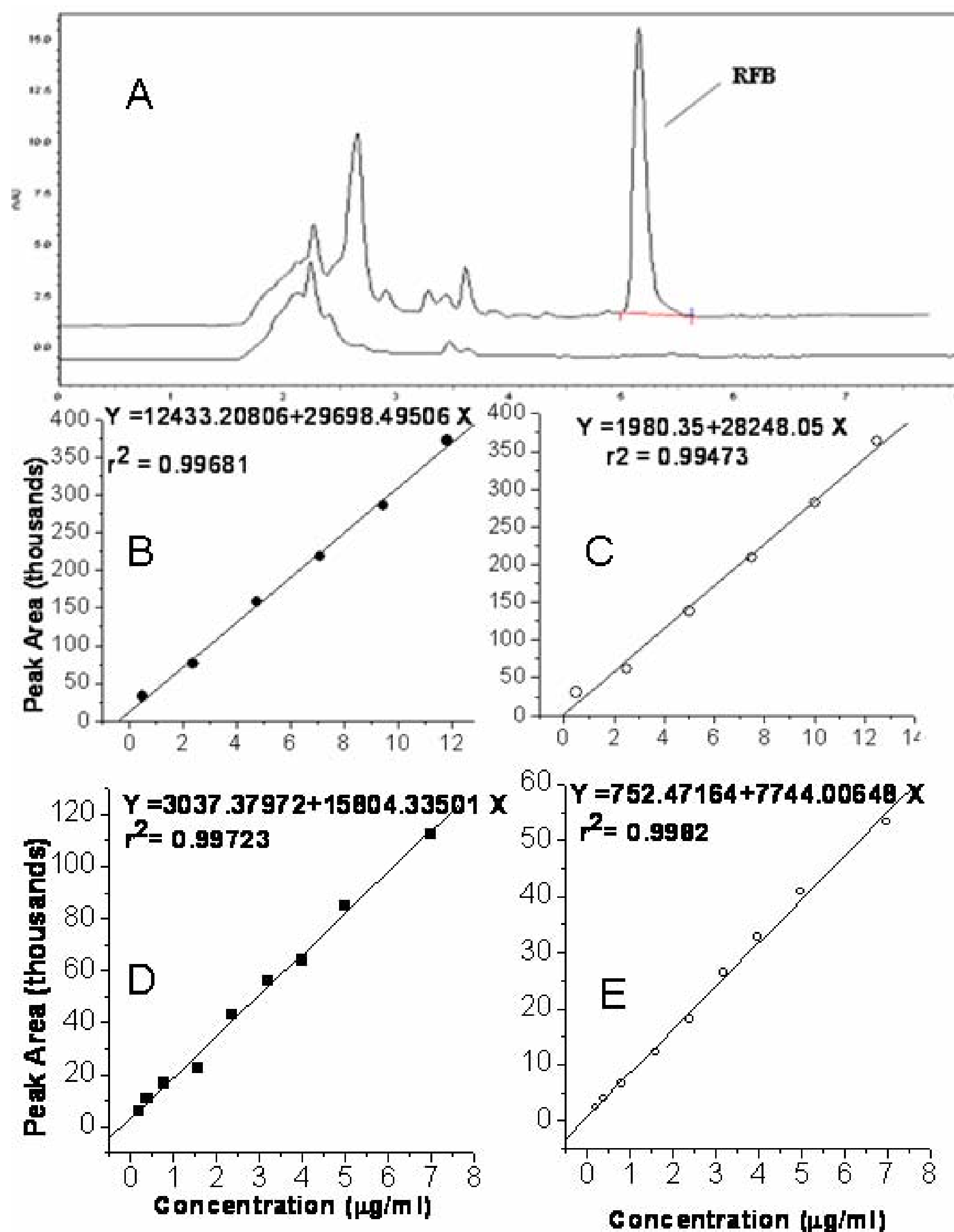

Figure 2: Quantitative analysis of rifabutin. The chromatograms (A) show the elution of the baseline-resolved peak at ~12 minutes (upper trace), with no interference from serum extractives in the vicinity (lower trace). Calibration curves with analytical standard (B) and working standards (C) prepared for the cotton wool matrix were satisfactory. Analytical standard (D) and working standard (E) plots were also satisfactory when biomatrix was analyzed.

Figure 3

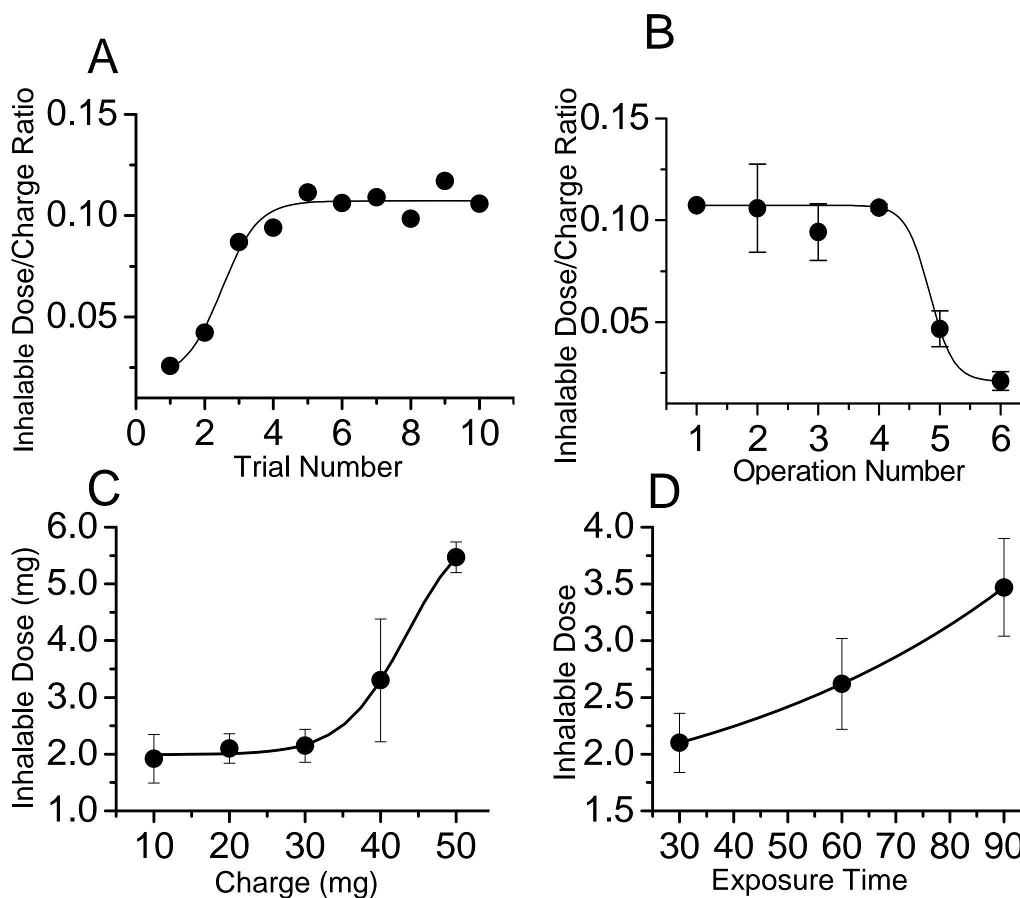

Figure 3: Standardization of the performance characteristics of the inhalation apparatus. (A): The dose available for inhalation as a function of charge was initially low, but showed saturable enhancement when the walls of the tube were 'primed' with microparticles. On saturation, there was negligible variation in the dose available for inhalation as a function of the charge. (B): Multiple dosing for 30-second periods after a single charge of 20 mg result in rapid depletion of the dose available for inhalation. The weighted sigmoidal fit was good, indicating the possibility of administering inhalations to 3 animals after a single charge of 20 mg, but the χ^2 value was only 0.495. (C): The dose available for inhalation did not change with small differences in charge, exhibiting stable values between 10 and 30 mg of charge. (D):

Duration of operation affected the dose available for inhalation. All determinations were carried out by HPLC. Gravimetric determinations showed concordance of 83 to 95% with the HPLC data.

Figure 4

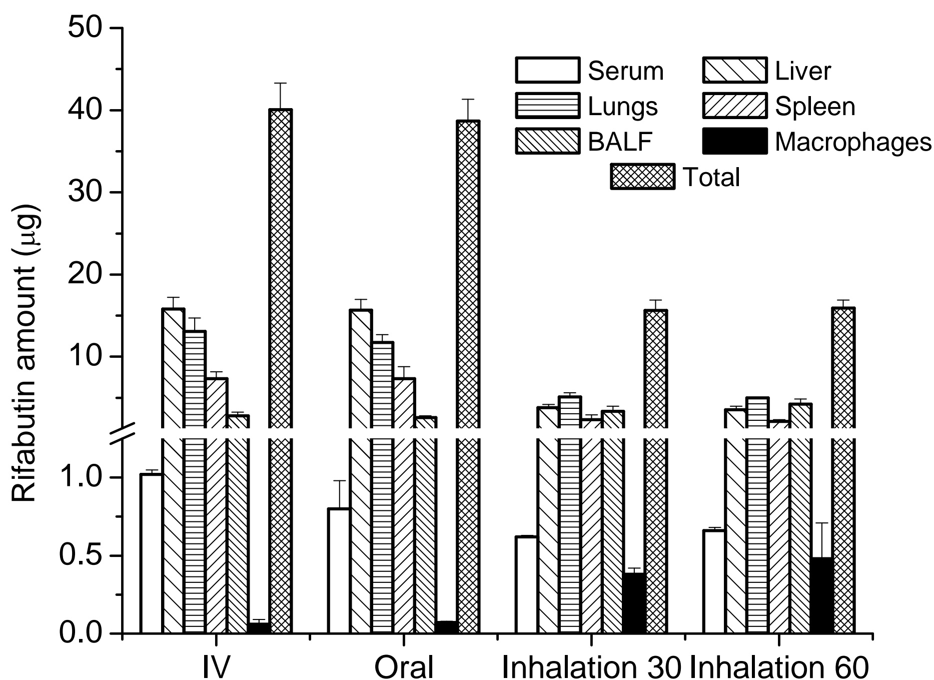

Figure 4: Rifabutin biodistribution following administration of 83 µg by intravenous injection, the same amount by oral gavage, and by inhalation over 30 sec with a charge of 20 mg in the apparatus.

Table 1: Projected amounts of rifabutin in various tissue compartments after [21]. Figures in bold face represent direct determination by HPLC.

Tissue	Intravenous: 83 μg		Oral: 83 μg		Inhalation: ~2 mg, 30 sec		Inhalation: ~2 mg, 60 sec	
	Amount (μg)	SD	Amount (μg)	SD	Amount (μg)	SD	Amount (μg)	SD
Serum	**1.02**	**0.02**	**0.80**	**0.18**	**0.62**	**0.01**	**0.67**	**0.02**
Adipose tissue	13.42	0.32	10.54	2.33	8.08	0.17	8.74	0.22
Kidney	8.54	0.20	6.64	1.47	5.09	0.11	5.50	0.14
Bone Marrow	8.45	0.20	6.64	1.47	5.09	0.11	5.50	0.14
Skin	5.74	0.14	4.51	1.00	3.45	0.07	3.74	0.09
Heart	4.57	0.11	3.59	0.08	2.76	0.06	2.98	0.08
Thigh muscle	2.26	0.05	1.78	0.39	1.36	0.03	1.47	0.04
Brain	0.49	0.01	0.39	0.08	0.30	0.01	0.32	0.01
Liver	24.67	0.58	19.38	4.30	14.85	0.31	16.07	0.39
	15.78	**1.42**	**15.68**	**1.31**	**3.82**	**0.37**	**3.57**	**0.40**
Lung	17.88	0.42	14.05	3.11	10.77	0.23	11.64	0.28
	13.08	**1.64**	**11.74**	**0.96**	**5.10**	**0.51**	**4.97**	**0.11**
Spleen	11.88	0.28	9.33	2.07	7.15	0.15	7.73	0.18
	7.34	**0.83**	**7.35**	**1.44**	**2.36**	**0.59**	**2.15**	**0.18**
Carcass	3.39	0.08	2.66	0.59	2.04	0.04	2.21	0.05
Total (Projected)	102.2 (118.79%)	2.41	80.29 (96.39%)	17.79	61.53	1.29	66.53	1.64