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Research Article

Application of a Four-fluid Nozzle Spray Drier to Prepare Inhalable Rifampicin-containing Mannitol Microparticles

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Abstract. The purpose of this study was to use a four-fluid nozzle spray drier as a new one-step method for preparing rifampicin (RFP)-containing mannitol microparticles. A RFP-acetone/methanol (2:1) solution and aqueous solutions of mannitol (MAN) were simultaneously supplied through different liquid passages of a four-fluid nozzle spray drier and then dried to obtain MAN microparticles containing RFP. Using a cascade impactor, the *in vitro* aerosol performance of RFP powder and RFP-MAN microparticles with 1:5, 1:10, and 1:20 ratios was compared. The *in vivo* retention of RFP in the lungs of rats after intratracheal administration of 1:20 RFP-MAN microparticles was also compared. The RFP-MAN microparticles had better aerosol performance than RFP powder and delivery to the lung stages improved as the fraction of MAN was increased. For the 1:20 RFP-MAN microparticles, deposition in stages 2–7 was approximately 43%, which is sufficient for treatment. Approximately 8% of the RFP-MAN microparticles were deposited in stages 6–7, which corresponds to alveoli containing alveolar macrophages. The initial retention of RFP in the lung following pulmonary delivery of 1:20 RFP-MAN microparticles was higher than following oral or intravenous administration of RFP, but the elimination was rapid, resulting in the disappearance of RFP from the lung within 4 h. The plasma concentration–time profile of RFP after intratracheal administration of 1:20 RFP-MAN microparticles was consistent with the profile for RFP retention in the lung. Addition of cholesterol or phosphatidylcholine to RFP had little effect on its retention in the lung. The RFP-MAN microparticles were effective for delivery of RFP to the lung, but the RFP rapidly removed from the lung into the blood circulation. This study demonstrated that RFP-containing MAN microparticles prepared in one step using the four-fluid nozzle spray drier efficiently deliver RFP to the lung, although methods must be developed to prolong its retention and improve targeting to alveolar macrophages.

KEY WORDS: four-fluid nozzle; inhalation; microparticles; spray dry; tuberculosis.

INTRODUCTION

There are various techniques for improving the solubility of water-insoluble drugs. Traditional methods for producing particles with enhanced solubility include the pulverization of large drug particles using a ball or jet mill [1–3]. Spray freezing and drying methods have been recently explored for preparing polymer-containing solid dispersion particles to enhance the dissolution rate of drugs [4–13]. However, the solid dispersion method requires a common solvent for the water-insoluble drug and the water-soluble polymer.

The four-fluid nozzle spray drier has a unique nozzle, with two liquid and two gas passages, which allows drug and carrier to be dissolved in separate solvents. This avoids problems of finding and using a common solvent for both drug and carrier. The four-fluid nozzle spray drier was previously used to generate two-drug composite microparticles and mannitol (MAN) microparticles containing polymeric or drug nanoparticles in one step [14–16]. The use of the four-fluid nozzle spray drier to prepare MAN microparticles containing nanoparticles of the water-insoluble compound pranlukast hemihydrate for oral, pulmonary, and injection dosage was also reported [17].

Globally, approximately two billion people are infected with tuberculosis (TB). This corresponds to one-third of the total population. In 2005, there were 8.8 million new cases of TB, and it resulted in the deaths of 1.6 million people, which is equivalent to an estimated 4,400 deaths per day [18]. The causative agent, *Mycobacterium tuberculosis*, infects the host lung following inhalation. Once in the lung, it is phagocytosed by alveolar macrophages, where it remains alive. Current treatment of TB involves the long-term oral administration of high doses of multiple drugs. This treatment can lead to serious side-effects, such as a liver damage. Furthermore, patient compliance is poor. Achieving an effective concentration of drug by oral administration and a complete cure is complicated by the difficulty of delivering drugs to the sites deep within the lungs where *Mycobacterium tuberculosis* resides. Several studies have attempted to improve the delivery of drugs into the lungs [19–33]. Such targeted

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delivery of drugs is expected to improve the treatment of TB, decrease the necessary doses, and reduce systemic side-effects.

Here, the four-fluid nozzle spray drier was used for the one-step preparation of rifampicin (RFP)-containing mannitol (MAN) microspheres for inhalation therapy of TB. The *in vitro* aerosol performance of the microspheres and the retention of RFP in the lung following their delivery *in vivo* were characterized.

MATERIAL AND METHODS

Materials

RFP (Sigma-Aldrich, St. Louis, MO, USA) was used as an anti-tuberculosis drug. The solubility to water of RFP at 20°°C is 1.3 mg/mL at pH 4.3 and 2.5 mg/mL at pH 7.3. MAN (Wako Pure Chemical Industries, Osaka, Japan) was used as a carrier for microparticles in which RFP was dispersed. Cholesterol (Chol; Wako Pure Chemical Industries, Osaka, Japan) and phosphatidylcholine (PC; Sigma-Aldrich) were used as lipids. A 5× Cell lysis reagent (Promega, Madison, WI, USA) was used in *in vivo* studies.

Preparation of RFP-containing MAN Microparticles using a Four-fluid Nozzle Spray Drier

Spray-dried particles were prepared using a model MDL-050 four-fluid nozzle spray drier (Fujisaki Electric, Tokushima, Japan). Figure 1 depicts the four-fluid nozzle, which has two chisel-shaped nozzles through which compressed air passes and two additional nozzles through which sample solutions pass. Different liquids and gases can be supplied to each passage individually. The sample solutions are withdrawn by high-speed compressed air in the acceleration zone. Air collides at the tip of the edge, and a powerful shock wave is generated at the collision focal spot. As a result, the drawn solutions are atomized into droplets. The droplets are then dried by heated air, and the dried particles are collected.

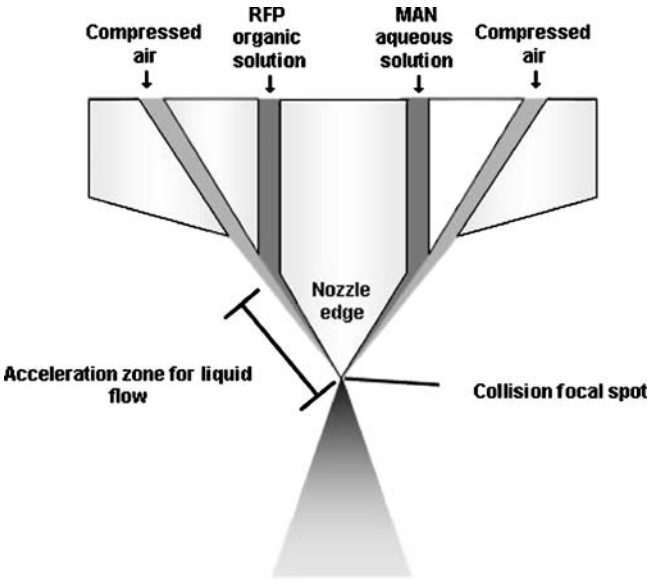

Fig. 1. Schematic diagram of the four-fluid nozzle

RFP was dissolved at 3.33%, 1.67%, and 0.83% (*w*/*v*) in a 1:1 solution of acetone/ethyl acetate, and MAN was dissolved in water at 16.7% (*w*/*v*). This gives RFP/MAN composition ratios of 1:5, 1:10, and 1:20 (*w*/*w*). When lipids were added, RFP, Chol, and PC were dissolved at 0.83%, 0.415%, and 0.415% (*w*/*v*), respectively, in a 1:1 solution of methanol/dichloromethane, and MAN was dissolved in water at 16.7% (*w*/*v*).

The RFP or RFP + lipid solutions and the aqueous solution of MAN were supplied through different liquid passages of the four-fluid nozzle. Spray drying was performed under the following conditions: inlet temperature, 40 °C; outlet temperature, 22 to 30 °C; supply rate for RFP or MAN solutions, 5 mL/min; spray rate for air, 30 L/min; spray air pressure, 0.78 MPa.

Scanning Electron Microscopy (SEM)

Particles were observed with an S-2250N scanning electron microscope (Hitachi, Tokyo, Japan). The samples were coated with 25-nm thick gold using a model SC-701 quick carbon coater (Sanyu Electronics, Tokyo, Japan).

Measurement of Mean Particle Diameter

The diameters of the microparticles (horizontal Feret diameters) were determined by image analysis from approximately 500–800 particles using WinROOF image analysis software (Mitani, Fukui, Japan). The mean particle diameter was defined as the median diameter of the cumulative curve of the number-basis particle size distribution.

Powder X-ray Diffraction Measurements

A Geigerflex RAD-IB powder X-ray diffractometer (Rigaku, Tokyo, Japan) was used to analyze the crystallinity of the samples. The conditions were as follows: target, Cu; filter, Ni; voltage, 40 kV; current, 20 mA; scan rate, $2\theta = 3°$/min.

In vitro Aerosol Performance

The *in vitro* aerosol performance of RFP from RFP powder or RFP-MAN microparticles was evaluated using an AN-200 Andersen nonviable sampler (Tokyo dyrec, Tokyo, Japan). The cascade impactor is listed in European Pharmacopoeia and United States Pharmacopoeia as equipment for evaluating inhalers [34–36]. The cascade impactor consists of a throat, a pre-separator, and eight stages (stages 0–7). Each stage consists of metal plate with small holes (nozzle) and a glass plate for collecting particles. An 8:1:1 methanol/glycerin/water solution was soaked into gauze and placed on the glass plates to prevent the falling of the particles deposited on the plates. A Jethaler® dual chamber type inhalation device (Hitachi, Gunma, Japan) was used as the inhalation device [37]. A hydroxypropyl methylcellulose capsule (size No. 2; Shionogi Qualicaps, Nara, Japan) was filled with 30 mg of RFP powder or RFP-MAN microparticles [38]. The inhalation test was performed at an inhalation rate of 28.3 L/min for 10 s. Prior to the inhalation study, the calibration of the flow rate was performed several times with the inhaler containing an empty capsule. After aspiration, the particles that deposited on the capsule, device, throat, pre-separator, and each

stage of cascade impactor were rinsed off with distilled water. The RFP solution for each stage was diluted with distilled water to 100 mL using a measuring flask. The quantity of the RFP in the collected solutions was assayed spectrophotometerically by measurement of the absorbance at 254 and 333 nm. The fine particle fraction, which is the total percentage deposition at stages 2–7 of the cascade impactor, was used to evaluate the aerosol performance. A higher fine particle fraction deposition is thought to indicate a higher *in vitro* aerosol performance.

In vivo RFP Residual Study in Lung

Sprague–Dawley male rats (8 weeks old; 250–300 g; Japan SLC Inc., Shizuoka, Japan) were used. The animals had free access to food and water according to the Guidelines of Experimental Animal Care issued by the Prime Minister`s Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Sciences. The rats were fasted for 1 day before the experiments. For lung delivery experiments, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital. The rat was placed in a holder with its upper incisors hooked onto the frame and the body positioned face up. The experiments were performed under incandescent lighting to maintain the body temperature of the rats. The middle of the fifth and sixth tracheal cartilages from the glandula thyroidea was cut, and a polyethylene tube (2.42 mm outside diameter×3 cm) was inserted 1.5 cm into the trachea for cannulation. RFP-MAN (1:20 RFP/MAN) or (RFP + lipids)-MAN ([RFP + lipids]/MAN = [1+1]/20) microparticles (2.5 mg/kg of RFP) were introduced by intratracheal administration using a veterinary dry powder insufflator (Model DP-4; Penn Century, Philadelphia, PA, USA) equipped with a three-way cock. Compressed air for releasing the particles was generated by depressing the plunger from 2 to 0.5 mL on the syringe scale. The delivery tube of the insufflator was inserted into the tracheal cannula and the rat's breathing was stopped for 2 s. Next, the air pulse was released through the three-way cock to deliver the particles, and the position was held for 5 s. The delivery tube was then removed from the cannula. For oral administration experiments, gelatin capsules (size No. 9; Torpac Inc., Fairfield, NJ, USA) were filled with RFP-MAN microparticles (10 mg/kg of RFP) and orally administrated to rats using a dosing syringe (Torpac). For intravenous administration experiments, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital. The rat was placed in a holder with its upper incisors hooked onto the frame and the body positioned face up. The experiments were performed under incandescent lighting to maintain the body temperature of the rats. RFP powder was dissolved in Tris buffer (pH 8.0) containing 0.1% Tween 80 to produce a solution of RFP (5 mg/mL of RFP). The RFP solution was administered through the femoral vein of a rat, resulting in a dose of 10 mg/kg of RFP.

The rats were exsanguinated 5, 60, and 240 min after administration, and their trachea and lungs were carefully extracted. The lungs were separated from the extract and homogenized in 6 mL of phosphate-buffered saline (pH 7.4) at 12,000 rpm for 1 min. The sample was centrifuged at 4,770×*g* for 1 min at 4 °C, and the supernatant was collected. Six milliliters of 5× cell lysis reagent was added to the residue, and the lysate was centrifuged at 4,770×*g* for 10 min at 4 °C. This supernatant was mixed with the supernatant collected in the previous step. Two hundred microliters of acetonitrile containing 0.02% (*w*/*v*) butylhydroxytoluene was added to 100 μL of the supernatant. After vortex mixing, the solution was centrifuged at 9,730×*g* for 5 min at 4 °C to remove the proteins. Two hundred microliters of ethanol containing 0.02% (*w*/*v*) butylhydroxytoluene was then added to 200 μL of the supernatant. After vortex mixing, the solution was centrifuged at 9,730×*g* for 5 min at 4 °C. The supernatant was evaporated to dryness using a HVC-500 mini-centrifugal concentrator (Asahi Technoglass, Chiba, Japan). The dry residue was dissolved in 200 μL of mobile phase, which was a 45:55 mixture of acetonitrile/mobile phase stock solution. The mobile phase stock solution was prepared as follows: monobasic phosphate (2.72 g), citric acid (3.23 g), and sodium perchlorate (1.08 g) were dissolved in 1,000 mL of ultrapure water and then mixed with 0.125 mL of phosphoric acid. Sixty-five microliters of the solution was analyzed by a high performance liquid chromatography (HPLC) to determine the plasma concentration of RFP. The HPLC conditions were as follows: pump, Hitachi L-7100; detector, Hitachi D-7400; integrator, Hitachi D-7500; column, YMC-Pack C8 (4.6 mm ϕ×100 mm; YMC, Kyoto, Japan); column temperature, 40°°C; detection wavelength, 333 nm; flow rate, 1.0 mL/min.

In vivo Pulmonary Absorption Study

Sprague–Dawley male rats (8 weeks old; 250–300 g; Japan SLC Inc.) were used. The rats were fasted for 1 day before the experiments. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital. The rat was placed in a holder with its upper incisors hooked onto the frame and the body positioned face up. The experiments were performed under incandescent lighting to maintain the body temperature of the rats. Carotid arterial cannulation was performed using a polyethylene tube (0.965 mm outside diameter). A syringe (1 mL) filled with heparin was placed at the end of the polyethylene tube through a 23-guage needle. The front of the neck of the rat was surgically opened along the median line. The middle of the fifth and sixth tracheal cartilages from the glandula thyroidea was cut, and a polyethylene tube (2.42 mm outside diameter×3 cm) was inserted 1.5 cm into the trachea for cannulation. RFP-MAN (1:20) microparticles (2.5 mg/kg of RFP) were introduced by intratracheal administration as described "*In Vivo* RFP Residual Study in Lung". Blood samples (400 μL) were taken from a carotid artery through the cannula. Plasma was obtained from the blood samples by centrifugation at 9,730×*g* for 15 min at 4 °C. The quantity of RFP in the plasma was measured by HPLC as described in "*In Vivo* RFP Residual Study in Lung".

RESULTS AND DISCUSSION

SEM Images and Physical Properties of RFP-MAN Microparticles

Figure 2 shows SEM images of RFP particles and RFP-MAN microparticles. RFP particles were a micronized

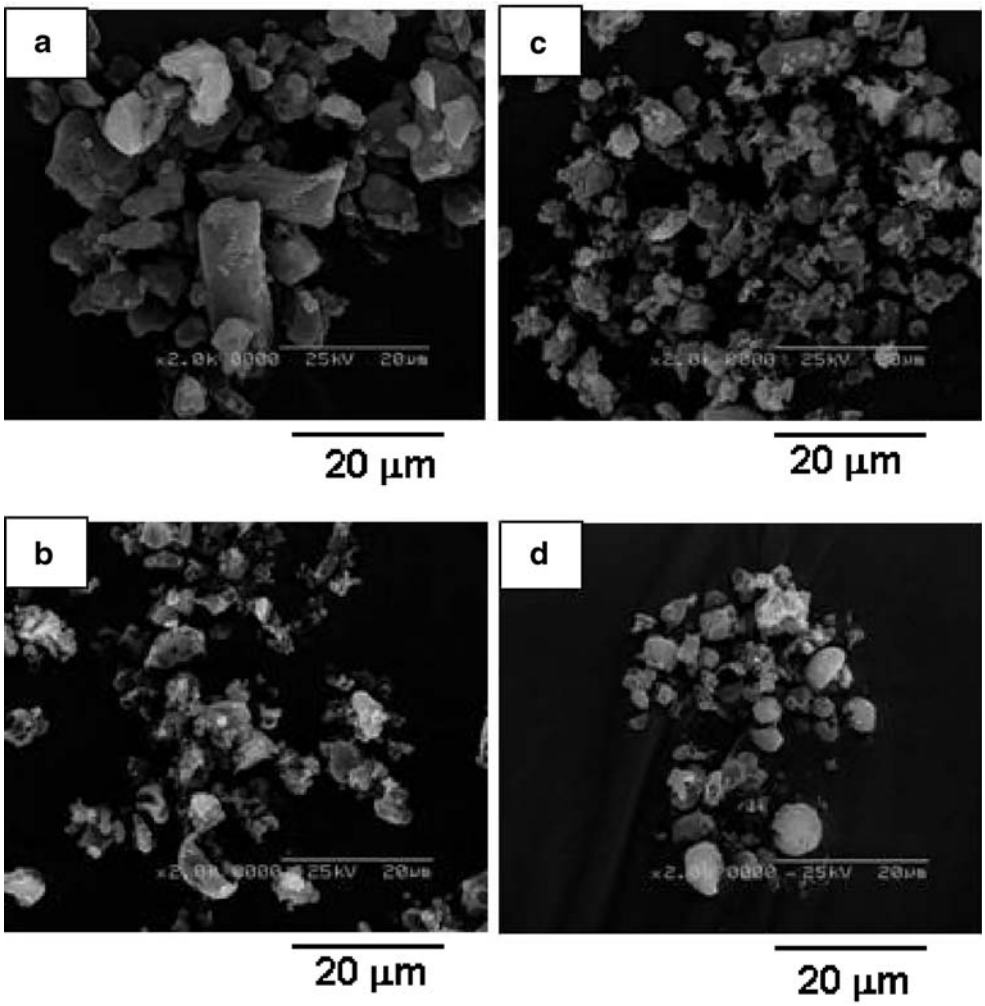

Fig. 2. SEM photographs of RFP **a** and RFP/MAN microparticles. RFP/MAN ratio (**b**, 1/5; **c**, 1/10; **d**, 1/20)

powder with a particle diameter between several and 10 μm. The RFP-MAN microparticles were more spherical in shape and had a high MAN ratio, and their mean diameters were 4.0, 3.8 and 3.2 μm at RFP/MAN ratios of 1:5, 1:10, and 1:20, respectively.

Figure 3 shows powder X-ray diffraction patterns of MAN, RFP particles, mixtures of RFP and MAN, and RFP-MAN microparticles at 1:5, 1:10, and 1:20 ratios. Peaks due to RFP crystals (*e.g.*, at approximately $2\theta=7°$ and 9°) were observed in the physical mixtures, but these peaks were not observed in the RFP-MAN microparticles, suggesting that the RFP was present in an amorphous state and/or in less perfect and smaller crystals in the microparticles.

In vitro Aerosol Performance of RFP Particles and RFP-MAN Microparticles

Figure 4 shows the *in vitro* aerosol performance of RFP particles and the RFP-MAN microparticles. In the case of RFP particles, approximately 33% and 31% of RFP remained in the throat and pre-separator, respectively. The deposition of RFP in stages 2–7 from RFP powder was less than 10% and nearly absent in stages 6 and 7. In contrast, for the RFP-MAN microparticles, deposition in the pre-separator was markedly reduced, and there was efficient deposition in stages 2–7, with approximately 43% deposition in these stages at a RFP/MAN ratio of 1:20. Furthermore, approximately 8% of the 1:20 RFP-MAN microparticles were deposited in stages 6–7. This result shows that it was possible to prepare the RFP-MAN microparticles using the four-fluid nozzle spray drier in one-step without the requirement of a common solvent for the poorly water-soluble RFP and the water-soluble MAN, and suggests that the dry powder containing RFP-MAN microparticles can be used for the inhalation treatment of TB.

In vivo Study of RFP Retention in the Lung After Intratracheal Administration of RFP-MAN or RFP + lipid-MAN Microparticles

The 1:20 RFP-MAN microparticles were delivered by intratracheal administration to rats and investigated the retention of RFP in the lung. For comparison, the retention after oral and intravenous administrations was also examined. In the case of oral administration, RFP was not present in the lung tissue after 1 h, and approximately 3% was observed after 4 h. In the case of intravenous administration, only 0.8% and 1.2% of RFP was observed in the lung after 1 and 4 h, respectively. In the case of pulmonary delivery, approximately 86% of RFP remained in the lung after 5 min; however, the

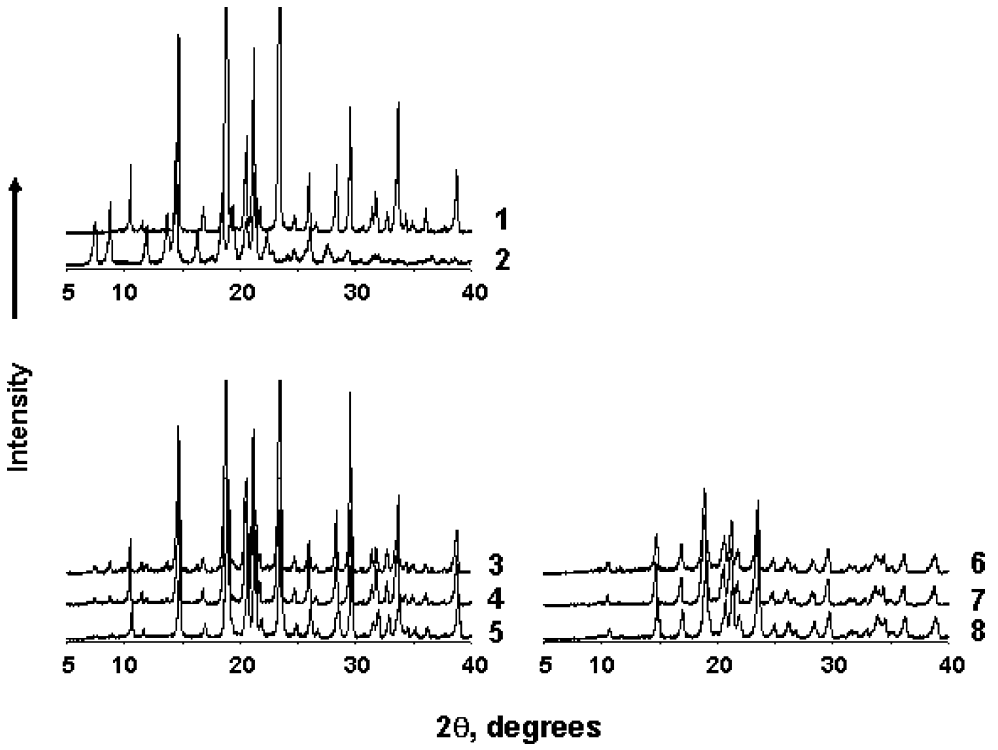

Fig. 3. Powder X-ray diffraction patterns of MAN (*1*), RFP (*2*), physical mixtures of RFP and MAN, and RFP/MAN microparticles. RFP/MAN ratio (*3* and *6*, 1/5; *4* and *7*, 1/10; *5* and *8*, 1/20)

residual amount decreased to only 4% after 1 h. Initial retention of RFP in the lung following pulmonary delivery was higher than that following oral and intravenous administration, but the elimination was rapid, and RFP disappeared within 4 h.

To investigate the cause of rapid elimination of RFP, the plasma concentration was measured after administration of the RFP-MAN microparticles. Figure 5 depicts the plasma concentration-time profile of RFP in rats after intratracheal administration of 1:20 RFP-MAN microparticles. The plasma concentration of RFP reached a maximum at 15 min, and no RFP was present in the plasma at 90 min. This result was consistent with that from the RFP residual study in lung. This result suggests that the RFP-MAN microparticles were effective for delivering RFP to the lung but that it was rapidly lost to the blood circulation.

Improvement of retention of RFP in the lung was tried by adding lipids (Chol and PC) to the RFP solution to delay the dissolution of RFP and to keep RFP on the surface of the lung, which resulted in (RFP + lipid)-MAN microparticles. For (RFP + lipid)-MAN microparticles, the residual percentage of RFP was high (~96%) 5 min after administration, but it fell to less than 2% after 1 h and less than 1% after 4 h. Therefore, addition of lipids to the microparticles did not

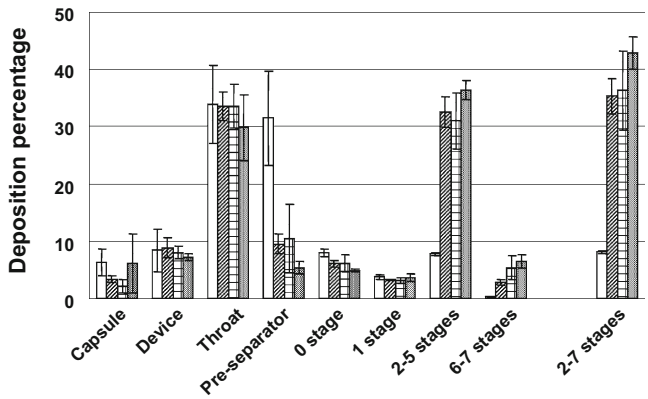

Fig. 4. *In vitro* aerosol performance of RFP particles and RFP/MAN microparticles. ☐, RFP particles. RFP/MAN microparticles (RFP/MAN ratio: ■, 1/5; ▦, 1/10; ▩, 1/20). Each point represents the mean ± SD (n=3)

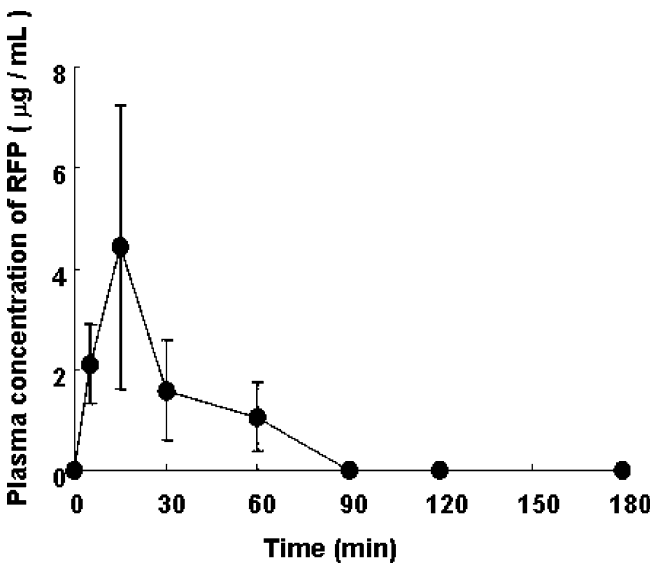

Fig. 5. Plasma concentration of RFP in rats after intratracheal administration of RFP/MAN microparticles at RFP/MAN ratio of 1/20. Dose: 2.5 mg/kg. Each point represents the mean ± SE (n=3)

result in the long-term retention of RFP in the lungs. The RFP was existed in an amorphous state and/or in less perfect and smaller crystals in the microparticles, as shown in Fig. 3, causing rapid dissolution of RFP after MAN was dissolved. The lipids used in this study could not suppress the dissolution of RFP and RFP was rapidly disappeared from the lungs.

CONCLUSIONS

This study demonstrated that RFP-containing MAN microparticles prepared in one step using the four-fluid nozzle spray drier without the requirement of a common solvent for RFP and MAN are efficient for delivering RFP to the lungs. However, technologies for prolonging the retention of RFP in the lung by suppressing the RFP dissolution from the microparticles using carriers and for efficiently targeting RFP to alveolar macrophages remain to be developed.

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