

## Evaluation of Aerosol Delivery of Nanosuspension for Pre-clinical Pulmonary Drug Delivery

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**Abstract** Asthma and chronic obstructive pulmonary disease (COPD) are pulmonary diseases that are characterized by inflammatory cell infiltration, cytokine production, and airway hyper-reactivity. Most of the effector cells responsible for these pathologies reside in the lungs. One of the most direct ways to deliver drugs to the target cells is via the trachea. In a pre-clinical setting, this can be achieved via intratracheal (IT), intranasal (IN), or aerosol delivery in the desired animal model. In this study, we pioneered the aerosol delivery of a nanosuspension formulation in a rodent model. The efficiency of different dosing techniques and formulations to target the lungs were compared, and fluticasone was used as the model compound. For the aerosol particle size determination, a ten-stage cascade impactor was used. The mass median aerodynamic diameter (MMAD) was calculated based on the percent cumulative accumulation at each stage. Formulations with different particle size of fluticasone were made for evaluation. The compatibility of regular fluticasone suspension and nanosuspension for aerosol delivery was also investigated. The *in vivo* studies were conducted on mice with optimized setting. It was found that the aerosol delivery of fluticasone with nanosuspension was as

efficient as intranasal (IN) dosing, and was able to achieve dose dependent lung deposition.

**Keywords** Lung inflammation · Intratracheal · Intranasal · Aerosol · Particle size · Pre-clinical · Impactor · Nano-suspension

### Introduction

Pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) and asthma are complex human airway diseases which affect millions of people worldwide. These human airway diseases are characterized by a strong lung inflammatory component with inflammatory cell infiltration, cytokine production, and airway hyper-reactivity.

Pulmonary disease populations are continuously increasing worldwide. More than 6% of the total US population suffered from asthma in 2004, up from a little over 3% in 1980. For these patients, glucocorticoids are often prescribed as first-line therapy to control symptoms, improve lung function, and reduce morbidity and mortality [1]. A treatment option for severe asthmatics is continuous or near continuous oral steroids (prednisolone). However, long-term use of high-dose ICS therapy has the potential to cause undesirable side effects. Side effects such as impaired growth in children, decreased bone mineral density, cataracts, skin thinning and bruising, glucose metabolism, and other hypothalamic–pituitary–adrenal-axis (HPA-axis) suppression effects are widely reported [1–5]. Multiple studies have demonstrated that the side effects of glucocorticoid therapy for human airway diseases are related to the systemic exposure of the drugs. More importantly, receptors responsible for efficacy in the lungs are also expressed in systemic tissues which are

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responsible for systemic side effects [6–9]. Due to the above findings, pulmonary targeting such as inhaled delivery is believed to provide an advantage over systemic administration, where the same degree of efficacy can be achieved with lower systemic exposure.

Aerosol pulmonary drug delivery is the preferred route of administration in the treatment of respiratory disease [10, 11]. Direct pulmonary delivery in humans is achieved using an aerosol generated by either an inhaler or nebulizer. The pharmaceutical industry is constantly seeking better and safer treatments for the pulmonary diseases. A major challenge in the identification of pulmonary drug candidate is demonstrating pre-clinical efficacy in appropriate animal models with good translatability to humans. In order to predict accurately the efficacy, delivery, distribution, and PK of an inhaled drug, it is necessary to simulate the characteristics of the drug aerosol in pre-clinical models. Intranasal and intratracheal delivery of test compounds are often used to deliver drugs directly to the lungs of experimental animals. However, the above methods are not practical in the clinical setting where an inhaler or nebulizer is used. Such a disconnect makes predicting PK/PD and efficacious dose in humans very difficult. A better pre-clinical modeling of drug delivery is necessary to obtain more accurate estimation of drug efficacy. In this study, aerosol delivery in an animal model was investigated and fluticasone propionate was used as a model compound. Fluticasone is a highly potent anti-inflammatory drug and is the most commonly prescribed inhaled glucocorticoid. A nanosuspension formulation ( $D_{50}$  0.2  $\mu\text{m}$ ) was evaluated against regular suspension ( $D_{50}$  1.6  $\mu\text{m}$ ) for aerosol delivery.

Nanosuspension has been widely used for drug delivery [12–16]. There are three key advantages of using nanosuspension formulations instead of solution formulations for pulmonary aerosol delivery. First, unlike solution formulations, nanosuspension formulations can be prepared without using large amount of organic co-solvents, thereby reducing the in vivo interference and potential toxicological effects caused by the co-solvents. Second, contrary to solution formations, the concentration of nanosuspension is not limited by solubility in the vehicle, thus, a wider dose range can be achieved. Finally, nanosuspension formulations provide superior content uniformity which results in greater confidence of delivery and reproducibility. Moreover, this mode of delivery can result in penetration of deep lung and smaller airways, leading to a more even drug distribution and resulting in a more accurate modeling of the drug distribution and efficacy.

In addition to the advantages conferred by nanosuspension formulations, there are several reasons to choose aerosol delivery as well. Importantly, aerosol delivery has been widely used and evaluated for pulmonary drug

delivery in humans. We believe combining nanosuspension and aerosol in the pre-clinical setting will enable better and more consistent results can be generated. This advantage should allow us establish a more accurate modeling of the drug distribution and the resulting efficacy. Despite the advantages of aerosol delivery, many factors can affect the outcome. Particle size, dosing system flow rate, characteristics of the nebulizer, and drug solution concentration can often affect the characteristics of the aerosol and the delivery efficiency. In general, particles  $<5 \mu\text{m}$  are considered suitable for inhaled drug delivery, and have a higher probability of being deposited in the airways of the lungs [17]. Despite these general understandings, conventional inhalers are found to be inefficient and highly variable in delivering the desired dose to patients [18]. Many factors have contributed to the above findings. Devices such as pressurized and ultrasonic nebulizers produce widely dispersed particle sizes and operate continuously, producing aerosol even when patients are not inhaling. This results in large transfer losses and poor lung deposition efficiencies [19] with the lung deposition being between 6 and 10% of the administered dose [20]. Despite the relatively low percent of deposition, the wide spread distribution of the particles in the lung results in equal or higher efficacy as compared to intratracheal or intranasal methods [21, 22]. In this study, we pioneer the pre-clinical use of nanosuspension for aerosol delivery. The effects of various parameters are optimized to generate an aerosol capable of delivering a suitable dosage to support pre-clinical studies in rodents. Lung deposition, plasma exposure, impact on particle size, and delivery techniques are evaluated.

## Materials and Methods

### Materials

Fluticasone propionate was purchased from Sequoia Research Products (Oxford, UK). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI), and reagent grade formic acid, sodium hydroxide obtained from EM Science (Gibbstown, N J). The HPLC system used for formulation potency check was an Agilent HP 1100 HPLC equipped with a diode array (DAD) and a variable wavelength UV (VWD) detectors, and a quaternary solvent delivery system (Palo Alto, CA). A Sciex API 4000 mass spectrometer (Applied Biosystems, Foster City, CA) coupled with HPLC was used for plasma drug analysis and quantification. Powder X-ray diffraction (PXRD) was done on a Bruker D-8 Advance diffractometer for all the solid-state works to confirm no form changes. A scintillation counter was used for detection. In-house-fabricated

aluminum inserts or inserts with a Hasteloy sintered filter (0.45  $\mu\text{m}$ ) pressed in the center and held in Bruker plastic sample cup holders were utilized for all the analyses. The water purification system used was a Millipore milli-Q system. All other chemicals were obtained from Aldrich (St. Louis, MO) and were used without further purification. A ten stage cascade impactor purchased from California Measurements (CA, USA) was used for aerosol particle size measurement. PARI LC jet nebulizer (Wuppertal, Germany) and BioAerosol Nebulizing Generator (BANG) from CH technology (NJ, USA) were used in our study. Jaeger-NYU Nose-Only Directed Flow Inhalation Exposure System from CH technology (NJ, USA) was used for aerosol animal dosing via a nose cone.

## Method

### *Formulation*

A bench scale wet milling (micronization) device was used [23] with an appropriate amount of glass beads to produce a nanosuspension formulation of fluticasone. Tween 80 at 0.5% (w/w) in phosphate buffered saline (pH 7.4) was added in a scintillation vial, and the mixture was stirred at 1,200 rpm for a period of 24 h with occasional shaking. The stock formulation was then harvested and potency was assessed by HPLC/DAD, and solid state checked by Powder X-ray diffraction (PXRD). Thermal gravimetric analysis with simultaneous differential thermal analysis (TGA/SDTA) was done on a Mettler TGA/SDTA851e. Particle size distribution was determined on a Beckman Coulter LS 230 particle size analyzer using the small volume accessory (Miami, FL). A PIDS obscuration water optical model was employed. Particle size distribution was computed by the software using Mie scattering theory. The large particle size ( $D_{50} = 1.6$  and  $D_{90} = 2.9 \mu\text{m}$ ) fluticasone for in vivo comparison work was purchased from Sequoia Research Products. The formulation was made by directly suspending the bulk drug in 0.5% (w/w) Tween 80 in PBS. Potency, homogeneity, chemical stability, and solid-state stability were performed following the same procedure as listed above.

### *Aerosol particle size measurement*

For the particle size distribution study, a ten-stage cascade impactor (GSI, California Measurement, Sierra Madre, CA., USA) was interposed between the nebulizer and the pump. The aerosol was generated using dry air at 50 psi. The impactor sampled the aerosol particles with aerodynamic diameters of 8.0 and 0.125  $\mu\text{m}$  at a continuous flow rate of 1 L/min<sup>-1</sup>. After a predetermined duration, the impactor was dismantled and different stages of the

cascade impactor were accurately weighted to determine the amount of deposition. The mass median aerodynamic diameter (MMAD) was calculated according to the percent cumulative accumulation at each stage.

### *In Vivo and Sample Analysis*

The Pfizer Institutional Animal Care and Use Committee (IACUC) reviewed and approved the animal use in these studies. The Association for Assessment and Accreditation of Laboratory Animal Care, International fully accredits the Pfizer animal care and use program. Male Balb/c mice [8–10 weeks, ~20 g] were used for all studies. For both the intratracheal (IT) and intranasal (IN) groups, the animals were anesthetized with an IP injection of a Ketamine/Xylazine mixture prior to the administration of 1 mg/Kg of Fluticasone in a nanosuspension and regular suspension. In the IT group, the anesthetized animals were held vertically with their mouths open. The tongue was pulled out gently to expose the trachea. Twenty-five microliters of the dose solution was pipetted into the trachea. For the IN group, the anesthetized animals were held vertically, and 25  $\mu\text{L}$  of the dose solution was pipetted dropwise to the naris until it was completely inhaled. For the aerosol dosing arm, the conscious animals were exposed to the aerosol in a nose-only system for 10 min. The animals were sacrificed with an IP injection of an overdose of Ketamine/Xylazine immediately after dosing. A blood sample was collected through cardiac puncture.

Plasma samples were obtained by centrifugation at 8,000 rpm for 10 min, and 50  $\mu\text{L}$  of the plasma sample was extracted with 100  $\mu\text{L}$  of acetonitrile containing 0.05  $\mu\text{M}$  of the internal standard (prepared in house). Samples were allowed to settle, and 50  $\mu\text{L}$  was transferred to a 96-well plate. Analytical standards were prepared by spiking known amount of standards into control plasma and according to the above extraction procedure.

Whole lung samples were collected, weighed, and kept on ice. In general, 200 mg of lung samples were ground in diluent containing 70% ACN and 30% water with the addition of 0.05  $\mu\text{M}$  of internal standard (made in house) to a final volume of 3.0 mL. Samples were allowed to settle, and 50  $\mu\text{L}$  was transferred to a 96-well plate. Analytical standards were prepared by spiking known amounts of standards into blank lung tissue according to the above extraction procedure. The concentration of fluticasone in all the samples was determined by LC/MS/MS on a Sciex API 4000 mass spectrometer in positive electrospray mode and MRM transition ( $m/z$  502.1/313.7). Limit of detection (LOD) was 0.00015  $\mu\text{g/mL}$ , and limit of quantification (LOQ) was 0.0006  $\mu\text{g/mL}$ .

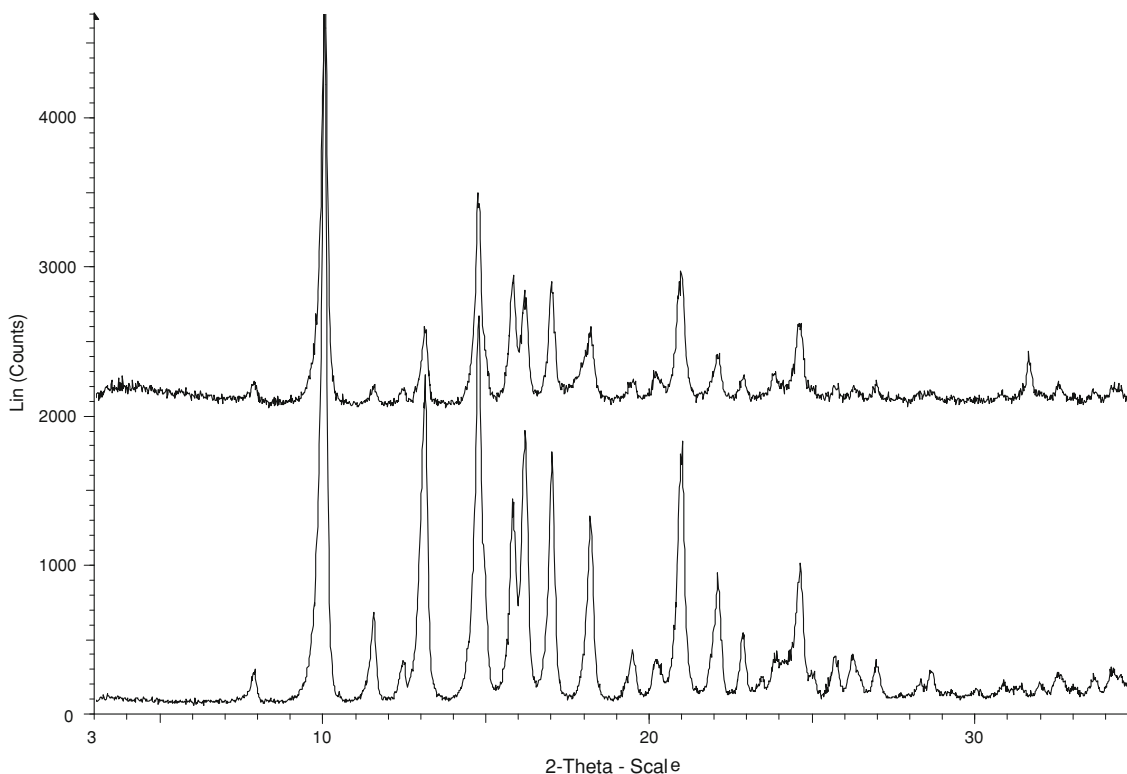
*Compatibility of regular and nanosuspension of fluticasone for aerosol delivery* These experiments were conducted

to determine the dose-dependent delivery to the lungs. Different concentrations of the fluticasone nanosuspension were nebulized. Conscious mice were loaded into restraining tubes, which left their naris exposed. The tubes were then attached to a Jaeger-NYU Nose-Only Directed Flow Inhalation Exposure System (12 port) via the nose cone, so that the naris of the animals were directly in front of a designated delivery vent. The dosing duration for animals in all aerosol delivery groups was kept to 10 min and sacrificed according to the general *in vivo* procedure.

*Comparison of the efficiency of different dosing techniques* For comparison studies, the efficiency of two nebulizers, PARI LC and BANG, was compared. Unanesthetized mice were loaded into restraining tubes, which left their naris exposed. The tubes were then attached to a Jaeger-NYU Nose-Only Directed Flow Inhalation Exposure System (12 port) via the nose cone, so that the naris of the animals were directly in front of a designated delivery vent. The flow rate, duration of dosing, dose solution concentration were optimized and adjusted according to the dosage required. The dosing duration for animals in all aerosol delivery groups was kept to 10 min and sacrificed according to the general *in vivo* procedure.

## Results and Discussion

Micronization of fluticasone successfully reduced the particle size of the bulk material from a mean value ( $D_{50}$ ) of 1.6 to 0.2  $\mu\text{m}$ . The solid form of the micronized material was examined by PXRD and TGA/SDTA, and demonstrated no discernable change in the crystal form post the micronization process. (Fig. 1). Content uniformity, potency, and homogeneity of the formulations were tested to ensure quality of test material for all studies. In general, nanosuspension performs very well in all the tests. Both nano and regular materials were dosed as suspension *in vivo*. Control samples (milled vehicle) were very clean with no glass shards observed. The dissolution rate was increased by reducing the particle size and was calculated by the Noye–Whitney equation. Solubility impact was calculated by the Oswald–Freundlich equation ( $\text{Log}(C_s/C_\infty) = 2\alpha V / (2.303RT\rho v)$ ) to further characterize the nanosuspension [24]. For fluticasone, a slight increase of solubility was observed (from 0.7  $\mu\text{M}$  to 1.1  $\mu\text{M}$ ) when particle size was reduced to 0.2  $\mu\text{m}$ . Despite the increase of solubility, greater than 99.9% fluticasone still exists as solid crystalline in nanosuspension. Thus, any particles formed from the supernatant during nebulization were ignored. Only aerosol particle from nanoparticle aggregation were considered.



**Fig. 1** Fluticasone PXRD patterns. Top is the post milling and bottom is the pre-milling API

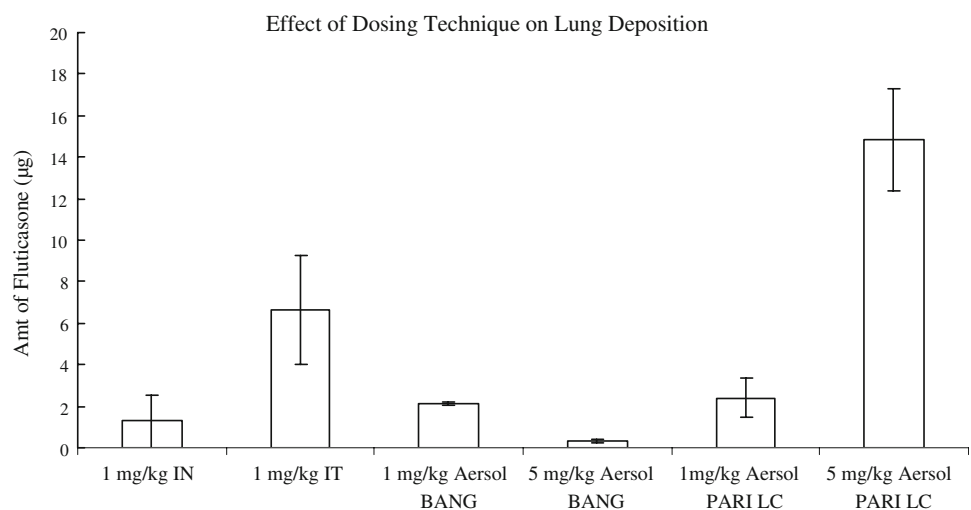
The first in vivo experiment was designed to compare different dosing techniques and impact of the nanosuspension on lung deposition and systemic exposure. The target was focused on higher and dose depended lung disposition (to enhance local efficacy) with lower systemic exposure (reduce systemic side effect). For this experiment, settings recommended by the manufacturer were used to test both PARI LC and BANG nebulizers. It was found that the PARI LC is more efficient at a higher drug concentration with the lung deposition of 2.4 and 14.9  $\mu\text{g}$  (approximately six folds) for the 1- and 5-mg/kg doses, respectively. The plasma exposures correspond to lung depositions with the evidence of reduced systemic exposures. For the PARI LC nebulizer, plasma concentration increase was observed when dose increased (for the 1- and 5-mg/kg doses: 0.01 and 0.02  $\mu\text{g}/\text{mL}$ ). However, the degree of increase is much smaller (only two folds) when compared with the lung exposure increase (approximately six folds). The dose-dependent increase in lung exposure along with lower systemic exposure was very much in accordance with the desired outcome. In comparison, the BANG device lost efficiency at a higher dose with a deposition of 2.0 and 0.3  $\mu\text{g}$  for the 1- and 5-mg/kg doses, respectively. The lack of delivery consistency at higher dose for BANG device was reflected in plasma level as well. For the 1-mg/kg dose the plasma concentration was determined at 0.01  $\mu\text{g}/\text{mL}$  and for the 5-mg/kg dose, plasma level was below the limit of detection. Based on the data, PARI LC nebulizer was chosen for further studies. The PARI LC device, provided a dose-dependent drug increase in lung disposition and evidence of reduced systemic exposure (compare with the IT dose). Detailed information is illustrated in Figs. 2 and 3.

Particle size distribution of the aerosol was further investigated by using a ten-stage cascade impactor with PARI LC nebulizer. The obtained data was used to further

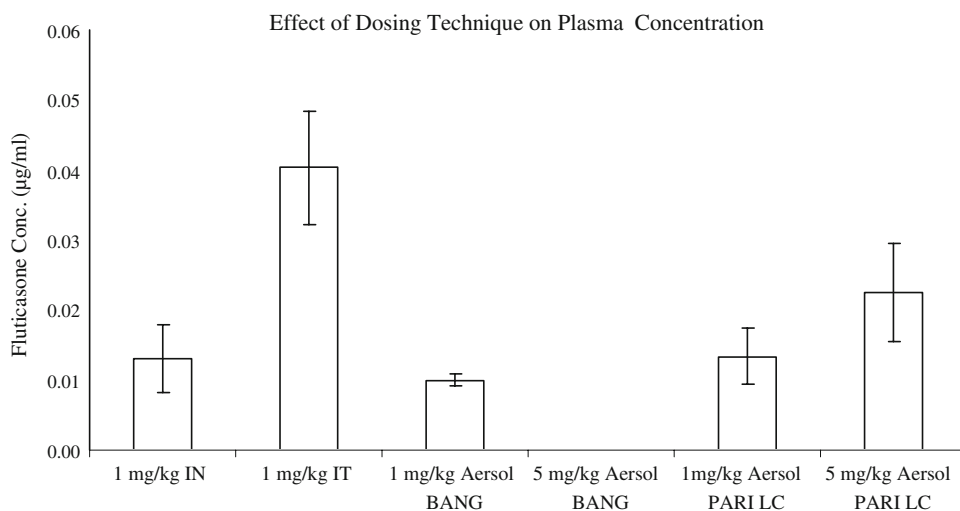
adjust the nebulizer to maximize the efficiency. Key parameters such as formulation concentration and system flow rates were investigated and found to impact the aerosol particle size and nebulizer efficiency. The system was optimized based on the best-obtained parameters. It was found when the optimized system was used, dose formulation, concentration, and flow rate had minimal effect on the aerosol particle size. The MMAD was obtained by linearly fitting the percent cumulative accumulation at each stage vs particle diameter. The obtained linear equations ( $y = ax + b$ ) were used to calculate the MMAD. In general, the MMAD of our optimized delivery setting was found to be about  $3.7 \pm 0.3 \mu\text{m}$  throughout a wide dose range (Table 1). The particle size of the nanosuspension formulation used for the studies is  $0.2 \mu\text{m}$  (D50). A MMAD of  $3.7 \pm 0.3 \mu\text{m}$  indicates that aerosol particles are indeed aggregates and each aggregate contains from 17 to 20 nanoparticles. The obtained MMAD is well within the respirable range of an aerosol. Furthermore, in this study, the MMAD ( $<5 \mu\text{m}$ ) of nanosuspension aerosol system is comparable to that of conventional aerosol system prepared from organic propellant [25, 26], which is clinically proven. Therefore, the delivery efficiency of nanosuspension is believed to be comparable to conventional systems. It is a viable option for pre-clinical drug delivery, and thus can provide a more suitable method for human dose projection.

Several in vivo experiments were adopted. The first experiment was designed to evaluate if regular suspension is suitable for in vivo aerosol delivery. The regular suspension was produced by using larger particle fluticasone (particle size D50 to D90  $1.6\text{--}2.9 \mu\text{m}$ ). The particle size range used in this study is within the range used for dry powder inhaler. In general, when larger particles were used for aerosol delivery, the fluticasone level was below LOD in both lung tissue and plasma. This finding was not a

**Fig. 2** Effect of dosing technique on lung deposition



**Fig. 3** Effect of dosing technique on plasma concentration



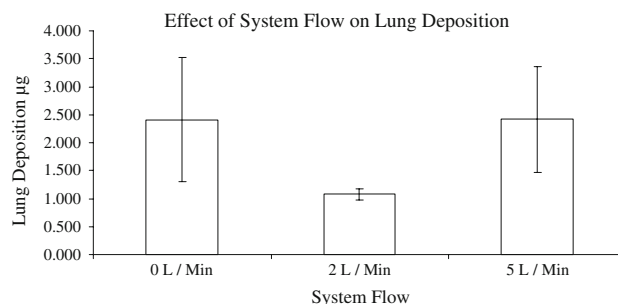
**Table 1** PARI LC Nebulizer MADD via Impactor for Nanosuspension on Different Dose Range

Formulation Concentration (mg/mL)	Duration (min)	MMAD (µm)
21.74	2.0	3.6
4.35	4.0	4.1
2.44	4.0	3.5
0.43	8.0	3.9
0.04	8.0	3.4
		Average 3.7 ± 0.3

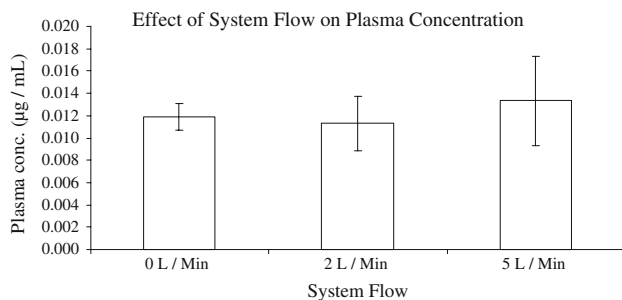
surprise. During the inhalation of an aerosolized drug, assuming if all the drug particles travel at a constant velocity, the large particles carry a higher momentum due to its increased mass. The higher momentum makes it difficult for these particles to negotiate the sharp turns in the anatomy of the nasal cavity and the transition to the upper airway. Thus, these larger particles tend to impact the inside of the nasal cavity or the back of the throat [27–29]. On the other hand, the smaller particles can change their trajectory with relatively increased ease, and can reach the lower parts of the airways [30]. Inside the deep lungs, the drug deposition is due to many factors which include Brownian motion, sedimentation due to gravity, and random impaction [31, 32]. Based on the number of nanoparticles found in each aggregate post nebulization, it is hypothesized that after nebulization, resulting aggregates from regular size particles were too large for inhalation. Due to the larger particle size used (particle size D50 to D90 1.6–2.9 µm), an aggregate with  $n > 4$  will result in a particle with  $D50 > 5$  µm which is considered too large for inhalation. When particles are larger than 5 µm, majority of the particles are trapped in the nasal cavity and upper airways, and only a small

percentage will actually reach the deep lung [33]. The majority of the fluticasone will then be deposited in the oral/nasal cavity and then swallowed. Because fluticasone is known to have very low oral absorption, low plasma exposure was expected. Based on the observation, it is concluded that regular size fluticasone suspension is not suitable for aerosol delivery. No further studies were conducted with regular size fluticasone.

The robustness of the delivery system was further tested in vivo. In this experiment, the impact of system flow rate on performance was further investigated. The flow rates tested for this study were 0, 2, and 5 L/min to cover the extreme cases, and the dose was set at 1 mg/kg. In general, our system was very robust. Using our delivery system, the lung depositions were not statistically significant, even under the extreme challenge. At a flow rate 0, 2, and 5 L/min, the lung depositions were 2.3, 1.1, and 2.4 µg/g, respectively (Fig. 4). Furthermore, the plasma concentration was about 0.01 µg/mL for all the groups (Fig. 5). If IT delivery is considered to be 100% on target delivery, the lung exposure via aerosol delivery is approximately 30%. In a clinical setting, the typical fraction delivered to lung is believed to be between 10 and 40% [9]. The amount of fluticasone deposited in the lung via nanosuspension aerosol delivery falls within the clinical range with low



**Fig. 4** Effect of system flow on lung deposition (PARI LC)



**Fig. 5** Effect of system flow on plasma exposure (PARI LC)

variability. This device provided a much better confidence in delivery in a relevant preclinical model in a range which mimics human exposure. Another highly desired advantage is reflected in the reduced systemic exposure. The systemic exposure from nanosuspension aerosol delivery is approximately 25% of the IT delivery. This low systemic exposure can provide a potential tool to differentiate topical (lung) vs systemic efficacy and side effects. This advantage is particularly important, because a major focus of pulmonary drug development is to improve local exposure/efficacy and minimizing systemic exposure and side effects. The lower systemic exposure observed via nanosuspension aerosol delivery will help researchers further explore the feasibility to differentiating topical (lung) vs systemic efficacy and side effects pre-clinically. Based on the results from various tests, we concluded that the aerosol delivery of fluticasone nanosuspension is very robust and well suited for pre-clinical pulmonary drug delivery. These novel studies demonstrate that combining nano-suspension and aerosol delivery is a valuable tool for pre-clinical pulmonary drug delivery. The major advantages of this delivery system include the absence of a propellant, ease of production, no solubility limit of the compound, and simulation of actual exposure in humans.

## Conclusion

In pulmonary drug discovery, the ability to evaluate new drugs for efficacy in animal model with quick turnaround and confidence of delivery is very important. In this study, we pioneered the usage of nanosuspension aerosol delivery in rodents, which is a system that is not limited by the solubility of the test compound. We have designed and validated a nanosuspension aerosol system that successfully delivers drug to the target (lung) in the pre-clinical animal model. Our data confirm that the system is suitable for pre-clinical drug delivery, and results from multiple conditions are highly repeatable and robust. These studies demonstrate that nanosuspensions combined with aerosol delivery could serve as a valuable tool for pulmonary drug discovery.

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