



Direct lung delivery of para-aminosalicylic acid by aerosol particles

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Summary Para-aminosalicylic acid (PAS), a tuberculostatic agent, was formulated into large porous particles for direct delivery into the lungs via inhalation. These particles possess optimized physical properties for deposition throughout the respiratory tract, a drug loading of 95% by weight and physical stability over 4 weeks at elevated temperatures. Upon insufflation in rats, PAS concentrations were measured in plasma, lung lining fluid and homogenized whole lung tissue. Systemic drug concentrations peaked at 15 min, with a maximum plasma concentration of $11 \pm 1 \mu\text{g/ml}$. The concentration in the lung lining fluid was $148 \pm 62 \mu\text{g/ml}$ at 15 min. Tissue concentrations were $65 \pm 20 \mu\text{g/ml}$ at 15 min and $3.2 \pm 0.2 \mu\text{g/ml}$ at 3 h. PAS was cleared within 3 h from the lung lining fluid and plasma but was still present at therapeutic concentrations in the lung tissue. These results suggest that inhalation delivery of PAS can potentially allow for a reduction in total dose delivered while providing for higher local and similar peak systemic drug concentrations as compared to those obtained upon oral PAS dosing. Similar particles could potentially be used for the delivery of additional anti-tuberculosis agents such as rifampicin, aminoglycosides or fluoroquinolones.

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Introduction

Large porous particles (LPPs), characterized by geometric sizes greater than $5 \mu\text{m}$ and mass densities less than 0.4g/cm^3 , have achieved recent popularity for drug delivery to the lungs for both

local and systemic applications.^{1,2} A principal advantage of LPPs relative to conventional inhaled therapeutic aerosol particles is their aerosolization efficiency.^{3,4} This permits the delivery of large drug masses (several tens of milligrams per puff) from a simple inhalation device.¹ In addition, these particles possess the potential for avoidance of alveolar macrophage clearance due to their large geometric size,^{5–7} enabling sustained drug release in the lungs.⁸ These traits suggest that inhalation of LPPs may provide an advantage in the treatment of tuberculosis (TB) by targeting the drugs directly to

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the primary site of infection in order to achieve therapeutic local drug concentrations with less systemic exposure than oral dosing. Inhalation delivery in general has been demonstrated to possess several advantages for TB treatment compared to oral delivery in theory⁹ and in practice.¹⁰ Since the lungs are directly targeted, total body doses are lower, leading to a decrease of potential drug resistance build-up. In addition, by avoiding the gastro-intestinal tract, side effects often associated with high oral doses (e.g. up to 12 g daily for para-aminosalicylic acid (PAS))¹¹ can be avoided. These arguments suggest that inhalation delivery of PAS by dry LPPs could, if proven equally efficacious as oral dosing, help to increase patient compliance, while increasing local concentrations of the drug with a practical and convenient inhalation system.

While previous researchers have conducted animal and human studies to explore pulmonary drug delivery for TB treatment,^{12,10} these systems involved liquid (nebulized) delivery. Nebulization is time consuming and cumbersome relative to standard therapies for asthma and COPD, which typically employ pressurized metered dose inhalers and dry powder inhalers. This article presents results for dry powder LPP delivery of a specific drug, PAS, commonly used to treat cases of multidrug resistant TB.¹³ We have formulated LPPs containing 95% of PAS by weight (LPP-PAS), confirmed that these formulations possess physical and aerosol properties suitable for use for the treatment of TB via inhalation and delivered them to rats via pulmonary insufflation to assess drug concentrations in plasma, lung lining fluid (assayed via bronchoalveolar lavage—BAL), and lung tissue homogenate.

Materials and methods

Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, MW = 734.05, purity $\geq 99\%$) was purchased from Genzyme Corp. (Cambridge, MA). PAS in the free acid form was purchased from Spectrum Chemical, Co. (New Brunswick, NJ). Distilled water USP grade was purchased from B. Braun Medical, Inc. (Irvine, CA) and ethanol USP grade was purchased from PharmCo (Brookfield, CT).

Particle production via spray drying

Solutions for spray drying were prepared by dissolving 0.25 g of DPPC and 4.75 g of PAS in 800 ml of ethanol. Solutions were protected from

light using aluminum foil. All solutions were used immediately after preparation. Solutions were spray dried utilizing a NIRO Atomizer Portable spray-drier system (Columbus, MD) equipped with a two-fluid nozzle for atomization. The 800 ml ethanol solutions were each mixed with 200 ml of distilled water immediately prior to atomization. Spray-dried LPP-PAS powders were collected via a cyclone.

Characterization of the spray-dried powders

The volume median geometric diameters (d) of two representative batches of the LPP-PAS powders were measured by light diffraction (Rodas, Sympatec, Lawrenceville, NJ) at an applied pressure of 1 bar.

A two-stage Anderson Cascade Impactor (ACI-2, Thermo Andersen, Smyrna, GA) was used to determine the fine particle fraction (FPF) of the LPP-PAS powders. In practice, a capsule containing approximately 10 mg of powder was placed in a hand-held, dry-powder, breath-activated inhaler device (the AIR[®] inhaler⁴). The capsule was punctured, a pump was actuated to simulate an inspiration (utilizing an air flowrate of 60 l/min for a duration of 2 s) and the powder was deposited on different stages covered with glass fiber filters depending on the aerodynamic diameter of the particles. The filters were weighed before and after the experiment; the fractions of particles with an aerodynamic diameter below 5.6 and 3.4 μm were determined from the deposited weights. The mass mean aerodynamic diameter (MMAD), written d_{aer} , is related to the geometric diameter d (assuming particle sphericity) by the formula¹⁴: $d_{\text{aer}} = d\sqrt{\rho}$, where ρ is the particle density normalized by the density of water (1 g/cm³).

Scanning Electron Microscopy (SEM) was performed using a LEO 982 (LEO Electron Microscopy, Inc., Thornwood, NY) operating between 1 and 5 kV with a filament current of approximately 0.5 mA. LPP-PAS powder samples were deposited onto SEM stubs covered with adhesive tape and then coated with a gold-chromium layer using a Polaron SC7620 sputter coater operated for 90 s at a sputtering current of 18 mA.

Accelerated physical stability

LPP-PAS powder samples were placed into a 40°C oven at 15% relative humidity to test accelerated physical stability. FPF and d were measured at 0, 2 and 4 weeks.

Animal experiments

This research adheres to the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1996). Male Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY). The animals weighed between 357 and 534 g (mean weight was 431.56 ± 7.29 g). The animals were in good health upon arrival and remained so until use; no clinical signs of illness were observed at any time. They were housed two per cage while on study in accordance to NIH guidelines prior to surgery in standard plastic shoebox cages with heat-treated laboratory grade Bed-O' Cobs (Anderson, Maumee, OH) bedding. The light/dark cycle was 12/12 h. The temperature in the animal room was ambient room temperature of approximately 25°C and the ambient humidity was in the range of approximately 35–60%. Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals (ILAR). Animals were allowed access to food and water ad libitum throughout the duration of the study. The food was Lab Diet-Rodent Diet #5001 (PMI Nutrition International, Inc., Brentwood, MO). The water was from a clean tap source from the City of Cambridge, Massachusetts Municipal Water Supply.

Indwelling jugular catheters were inserted under general anesthesia using a ketamine (80–90 mg/kg) (Fort Dodge, Inc., Fort Dodge, IA) and xylazine (10–13 mg/kg) (Phoenix Pharmaceuticals, St. Joseph, MO) cocktail by intra-peritoneal injection 24 h prior to dosing. The catheterization method was performed per an approved procedure.¹⁵ Following surgery, the study subjects were numbered upon pre-dose blood collection and placed into individually numbered cages overnight. The animals were used as presented in Table 1 for the different types of samples. Five-milligram samples of LPP-PAS powders were weighed out into hydroxypropyl-methyl cellulose capsules (Shionogi, Japan) and stored at 4°C prior to dosing. Following catheterization and prior to treatment, animals were distributed equally based on body weight throughout the treatment groups. Animals were anesthe-

tized by intraperitoneal injection of ketamine and xylazine as described above. The LPP-PAS powders were administered via an insufflation device (PennCentury, Philadelphia, PA). Once sedation was confirmed, animals were placed onto a modified slant board to optimize visual placement of the insufflation cannula into the trachea. The tip of the insufflation device was placed in the trachea just above the carina and 5 mg of LPP-PAS powder was delivered through the insufflation device by rapidly pushing a 3 cm³ bolus of air through the device a total of 3 times. Pharmacokinetic sampling time started immediately after the final 3 cm³ bolus of air was injected. All blood samples were taken via the implanted jugular catheter. Blood was collected pre-dose, and at 15, 30, 180, and 420 min following pulmonary insufflation. Approximately 0.4 ml of blood was taken at each time point. All blood samples were collected in EDTA tubes. Plasma was separated in a tabletop centrifuge (Eppendorf 5415C, VWR, Boston, MA) at 14,000 rpm for 30 s. The retrieved plasma was subsequently snap frozen on dry ice and stored at –80°C until analysis.

For BAL to assess lung lining fluid PAS levels, animals were euthanized via intraperitoneal injection of 216 mg of euthasol (Delmarva, Midlothian, VA) and exsanguinated per an approved IACUC protocol. The trachea was cannulated using an 18-gauge needle adaptor for subsequent injection and retrieval of broncho-alveolar lavage (BAL) fluid. Prior to BAL, the left main bronchus was clamped and subsequently tied off to isolate the large left lobe for whole tissue analysis. BAL was performed on the remainder of the lung (lavage buffer: 145 mM NaCl (8.5 g/l), 5 mM KCl (0.37 g/l), 1.9 mM NaH₂PO₄ (0.26 g/l for monohydrate; 0.228 g/l for anhydrous), 0.5 mM glucose (1 g/l) pH = 7.4). One ml of lavage buffer was slowly inserted via a 5 ml syringe and as much as possible of the fluid was retrieved for subsequent analysis. Retrieved BAL fluid was clarified by centrifugation at 250 RCF (1060 rpm) for 2 min. Following centrifugation, the supernatant was reserved, snap-frozen on dry ice, and stored at –80°C until analysis.

Table 1 Utilization of the rats for the different types of samples.

Group	# rats	0 min	15 min	30 min	180 min	420 min
1	5	Plasma	×	Plasma	Plasma/BAL lung	×
2	5	Plasma	Plasma	Plasma	×	Plasma/BAL lung
3	5	Plasma	Plasma/BAL lung	×	×	×

A total of 15 rats were insufflated in this study. At each timepoint, plasma samples were taken from a minimum of five animals. At terminal sacrifice timepoints (15, 30, and 420 min), BAL and whole lung homogenization were completed on a minimum of 5 sets of lungs.

Following BAL, the lungs were removed from the chest cavity for homogenization. The isolated left lobe was retrieved intact, weighed and placed into a 15 ml conical tube. The tissue was snap frozen on dry ice and stored at -80°C until homogenization. For homogenization, lung tissues were thawed and 3.0 ml of lavage buffer was added to each 15 ml tube. Lungs were homogenized using a Polytron PT-1200 Homogenizer (Kinematica AG, Luzernerstrasse, Switzerland). The subsequent tissue slurry was snap-frozen on dry ice and stored at -80°C until analysis. Plasma, BAL fluid and homogenized lung tissue PAS concentrations were determined by an HPLC assay described in the literature.¹⁶ Briefly, all samples were analyzed using high-performance liquid chromatography (HPLC) with the following equipment: a Waters model 510 pump and model 680 gradient controller and solvent select valve (Milford, MA), a Spectra Physics model 8875 fixed-volume autosampler (San Jose, CA), a McPherson model FL-750A fluorescence detector (Acton, MA), a Macintosh IIfx computer (Apple Computers, Inc., Cupertino, CA), and the Rainin Dynamax HPLC data management system (Woburn, MA). The standard curves for PAS were 1.0–100.0 $\mu\text{g}/\text{ml}$. The lower limit of detection was approximately 0.01 $\mu\text{g}/\text{ml}$, and the low standard was 1 $\mu\text{g}/\text{ml}$. The best fits of the standard curves were achieved using a weight of $1/Y^2$. The recovery of PAS from serum was 94% (range, 91–97%). Testing for PAS within sample precision produced a percent coefficient of variation (CV) of 2.0%. Testing for PAS day-to-day assay precision produced a coefficient of variation of 2.4% (low: 0.1% at 1 $\mu\text{g}/\text{ml}$; high: 5.0% at 10 $\mu\text{g}/\text{ml}$).

A total of 15 animals were insufflated in this study, five per treatment group. The time of terminal sacrifice was staggered to increase the pharmacokinetic and tissue sample size at each timepoint. For the PK studies according to Table 1, 15 animals were pre-bled to establish background levels. At 15 and 30 min post-dosing, 10 samples were collected and at the 180 and 420 min post-dosing timepoints, 5 samples were collected. To generate the PK data, a total of 5 samples were assayed from each timepoint. For BAL samples, at each terminal sacrifice, 5 samples were collected for each timepoint.

Results and discussion

Particle physical properties

SEM pictures reveal the particles to be porous and partly crystalline (Fig. 1, top). The geometric and

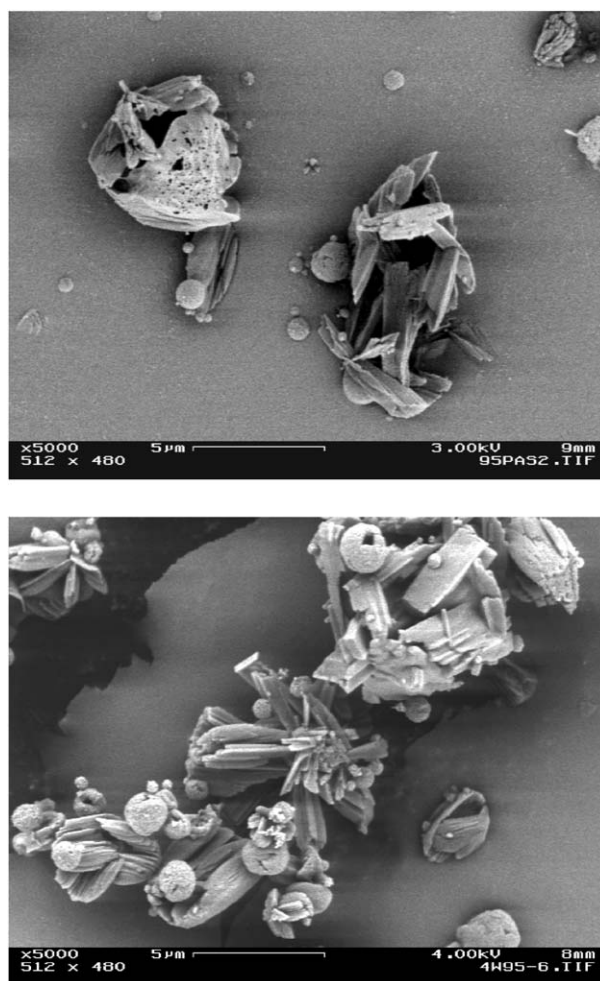


Figure 1 SEM images of the PAS LPPs just after spray-drying (top) or after 4 weeks at 40°C and 15% relative humidity (bottom). Scale bars represent 5 μm .

aerodynamic properties of two representative LPP-PAS batches are shown in Table 2; values of d were in the range of 7.0–7.5 μm whereas FPFs below 5.6 μm ranged from 0.59 to 0.63 and below 3.4 μm ranged from 0.31 to 0.39. These particle characteristics are typical of particles that deposit throughout the respiratory tract, with good deposition in the central airways and the alveolar region, based on human data for similar powders.¹⁷

Accelerated physical stability

Given the observation via SEM of the presence of crystalline phases in the particles (confirmed via DSC, data not shown), the stability of aerosol properties to physical changes was tested in an accelerated stability environment (40°C and 15% relative humidity). The geometric diameter at 1 bar and the FPF were tested at 2 and 4 weeks; neither

Table 2 Physical properties of the PAS particles for two different batches.

Batch	Volume median geometric diameter (d in μm)	FPF	
		<5.6 μm	<3.4 μm
1	7.47	0.596	0.311
2	7.07	0.629	0.392

Variability from batch to batch is acceptable for animal experiments since the insufflation method homogeneously delivers air powders to the lung.

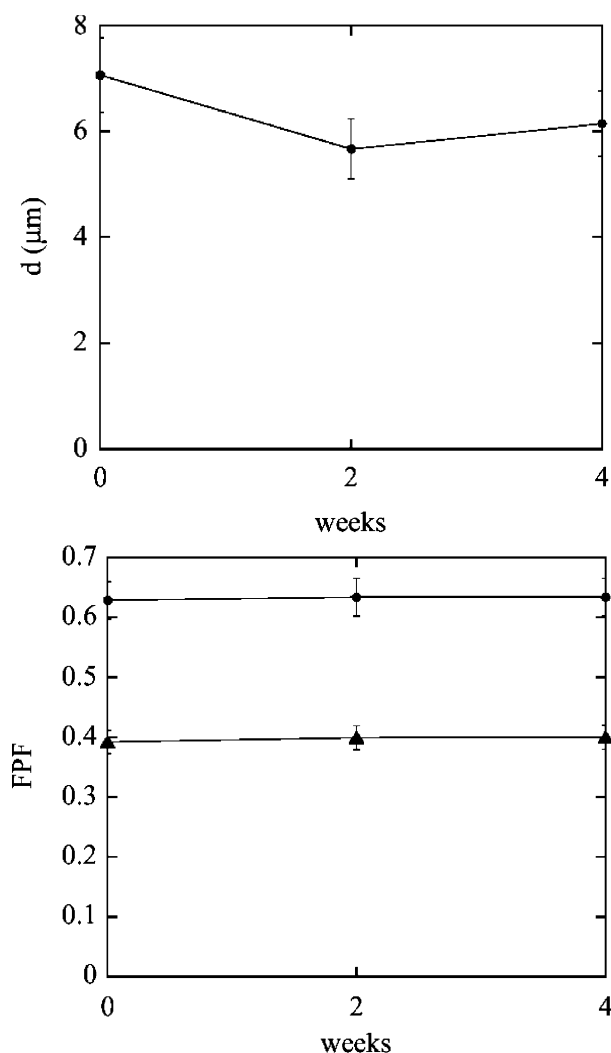


Figure 2 Geometric diameter at 1 bar (top) and FPF (bottom, ● represents $<5.6 \mu\text{m}$ and ▲ $<3.4 \mu\text{m}$) over 4 weeks at 40°C and 15% relative humidity.

displayed statistically significant changes (Fig. 2). Additionally, no observable increases or changes in crystalline content were observed over time with

SEM (Fig. 1, bottom). The PAS formulation thus appears to be physically stable over 4 weeks under elevated temperature exposure, an important feature for practical therapy.

Animal experiments

The pharmacokinetic data (Fig. 3) indicate that PAS delivered as 5 mg of LPP-PAS powder via insufflation reached the systemic circulation within 15 min of delivery to the lungs with a plasma peak concentration of $11 \pm 1 \mu\text{g/ml}$ (Table 3) and a time to peak concentration in the range of 0–30 min, with total clearance by 3 h post-exposure. For general comparison, the peak concentration that we observed in rats is an order of magnitude larger than the minimum inhibitory concentration (MIC) of PAS^{16,18} and in the same range of what is observed for humans upon oral dosing. Normalizing the inhaled PAS results for rat body mass, the total body dose is approximately 11 mg/kg via the lungs compared to an estimated value of 57 mg/kg for oral dosing of PAS in humans (assuming a single dose of 4 g administered to a 70 kg person).

Owing to limits of detection, we were not able to measure PAS concentration in the BAL fluid beyond 15 min. At 15 min, we found this concentration to be $5.7 \pm 2.4 \mu\text{g/ml}$. To attempt to estimate from this value the corresponding “true” lung fluid concentration (i.e. to account for the fact that the BAL buffer acts as a diluent), an estimated dilution factor was calculated. An estimate of the total lung lining fluid volume for a rat has been reported to be $80.2 \mu\text{l}$.¹⁹ Since only the right side of

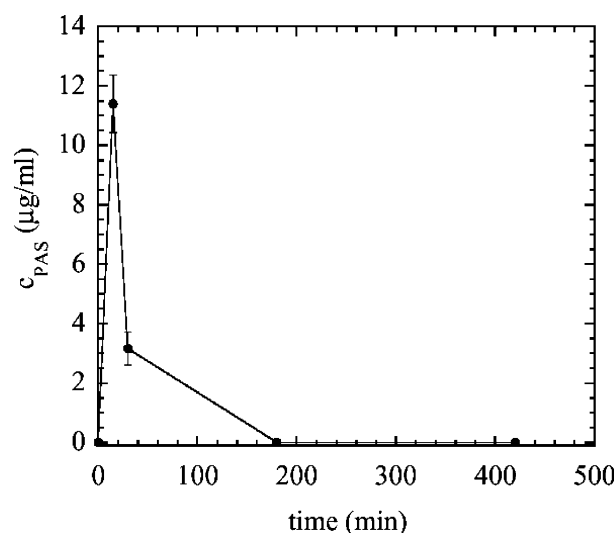


Figure 3 PAS pharmacokinetics in the plasma after insufflation of 5 mg of powder to rats. Data shown is mean \pm SEM of $n \geq 5$ guinea pigs at each timepoint.

Table 3 Measured and calculated PAS concentrations in plasma, lung fluid and lung tissue after insufflation.

	15 min ($\mu\text{g/ml}$)	180 min ($\mu\text{g/ml}$)
Blood	11 ± 1	0
Lung fluid	148 ± 62	0
Lung tissue	65 ± 20	3.2 ± 0.2

Data shown is mean \pm SEM of $n \geq 5$ guinea pigs.

the lung was lavaged, it was estimated that $40.1 \mu\text{l}$ of lung lining fluid was diluted in 1 ml of BAL buffer. Accounting for this dilution, the PAS concentration in the lung lining fluid at 15 min was calculated to be $148 \pm 62 \mu\text{g/ml}$ (Table 3), which is approximately thirteen times the measured PAS concentration in plasma at this time point.

The concentrations in the homogenized lung tissue were determined to be $3.3 \pm 1 \mu\text{g/ml}$ at 15 min and $0.16 \pm 0.01 \mu\text{g/ml}$ at 3 h. To estimate the dilution due to the buffer, the left lobe was removed, placed in 3 ml of buffer and homogenized: the new volume recorded was 3.16 ml. Thus the lung contribution to the volume is 0.16 and the dilution factor is $0.16/3.16 = 0.0506$. The concentration in the lung tissue is finally estimated to be $65 \pm 20 \mu\text{g/ml}$ at 15 min and $3.2 \pm 0.2 \mu\text{g/ml}$ at 3 h (Table 3). This indicates that, although not detectable in the blood or the lung fluid, PAS remains at therapeutic concentrations in the lung tissue for at least 3 h after insufflation.

Conclusion

We have formulated PAS, a tuberculostatic agent, into LPPs containing 95% PAS by weight via spray drying. These LPP-PAS formulations are for direct delivery to the lungs by inhalation. Their physical properties are optimal for deposition throughout the respiratory tract, from the central airways to the alveolar region, and these physical properties are stable at accelerated storage conditions.

The systemic and local concentrations observed after insufflation of LPP-PAS in rats suggest that greater lung exposure to PAS can be achieved at much less total body drug dose than those typically used for oral delivery. It is unclear what these pharmacokinetic data mean precisely in terms of the safety and efficacy of an inhaled PAS product, though we believe the results to be sufficiently

promising to argue for further dose-ranging safety and efficacy testing.

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