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A comparative study between high potency dry powder inhalation and nebulized solution of vancomycin hydrochloride for lung delivery

By

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A THESIS

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ABSTRACT

The main purpose of this research was to compare the *in vitro* deposition efficiency and efficacy of dry powder inhalations and nebulized solutions of vancomycin hydrochloride. Dry powder inhalations of vancomycin hydrochloride were prepared by spray drying solutions of VH in water at different concentrations (5 and 50 mg/mL) and at different spray flow rates (30-60mm) with other parameters constant. The spray dried (SD) powders were characterized by determining the moisture content, particle size, endothermic events and surface morphology of the powder. The spray drying process yielded dry powders that had residual moisture content consistent with that of the commercial powder before spray drying. The SD powders exhibited narrower particle size distributions compared to the commercial powder. VH did not show any change in its physical nature after the spray drying process and this was confirmed by the differential scanning calorimetry. Scanning electron microscopy revealed that SD particles were loose aggregates of porous spheres. Nebulized solutions of VH were prepared in normal saline (20 mg in 5 ml). The osmolality and pH of the VH solution was found to be within the acceptable limits set for nebulized solutions.

The SD powders (20mg) were filled in capsules for aerosolization using an Aerolizer Inhaler dry powder inhaler device while nebulized aerosols were generated using an Aeroneb Pro® vibrating mesh nebulizer. The *in vitro* aerodynamic profile of SD powders was determined using a Next Generation Impactor (NGI) at flow rate of 60L/min and compared to a flow rate of 15L/min for nebulized solutions. It was found that SD VH powders had smaller mass median aerodynamic diameters (MMAD) and higher
percentages of fine particle fraction (%FPF) and thus better in vitro deposition efficiency than the nebulized solution of VH.

The anti-bacterial efficacy of SD and commercial VH powder was determined using Methicillin-resistant Staphylococcus aureus (MRSA) cultures. The SD VH powders had equivalent antibacterial efficacy as compared to commercial VH powder, indicating that VH retained its therapeutic activity and that the spray drying process did not degrade VH. The research also established a proof of concept for an in vitro Next Generation Impactor (NGI) model which could be used for screening aerosolized antibiotics. A deep collection cup was custom made in order to accommodate the surface of bacterial cultures at a depth as specified in the USP. The NGI was operated normally but with the normal Stage 4 collection cup replaced by the modified deep collection cup. VH solution was nebulized at flow rate of 15L/min and the percentage of dose deposited in all the stages was determined with no statistically significant difference (p>0.05) in the deposited dose percentage at Stage 4 between normal and modified collection cups.

A MRSA culture was then incorporated in the modified collection cup and placed inside the NGI. The nebulization of VH solution (20 mg in 5 ml) resulted in complete inhibition of bacterial colonies in the bacterial culture plate. A proof of concept for an in vitro NGI model which could test the in vitro deposition efficiency and anti-bacterial efficacy of nebulized VH solution using a modified deep collection cup was successfully developed.
PREFACE

ABSTRACTS:

Dedicated to my parents
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It is a great privilege to express my deep sense of gratitude to my advisor Dr. Justin A. Tolman for his invaluable guidance, inspiration, constant encouragement and support during the tenure of research work and providing excellent research facilities. It has been an honor to be his first graduate student.

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<td>Mass median aerodynamic diameter</td>
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<td>% FPF</td>
<td>Percentage fine particle fraction</td>
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<td>GSD</td>
<td>Geometric standard deviation</td>
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<td>NGI</td>
<td>Next Generation Impactor</td>
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<td>Micro orifice collector</td>
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Chapter 1

Introduction
1. Lungs

The lungs are the primary component of the respiratory system and originate at the mouth and throat, include the trachea and bronchi, and undergo series of branching to finally terminate at the alveolar sacs. The airways are sometimes referred to as a pulmonary tree due to their overall resemblance to a tree. The tree trunk is analogous to the trachea of the airways. As the tree trunk divides into branches and ends in foliage, the trachea bifurcates into conducting tubes called bronchi and terminates in cluster of air filled sacs called alveolar sacs. (1)

1.1. Function and structure of lungs

The lung is designed to promote and optimize gas exchange. Lung physiology allows for the efficient movement of inspired air as well as diffusion of oxygen and carbon dioxide across thin alveolar membranes. Specifically, inspired air passes through the conducting airways: from the mouth and into the trachea which further divides into two main bronchi. The main bronchi further bifurcate into smaller bronchioles which lead into large physiologic segments called lobes; there are three lobes in the right lung and two lobes in the left lung. The smallest bronchioles, primarily involved in conduction of air, are called as terminal bronchioles with air movement and gas exchange occurring in the airway tubes distal to terminal bronchioles called respiratory bronchioles. The respiratory bronchioles further branch into alveolar ducts which finally terminate in alveolar sacs. The airways are divided into conducting airways and respiratory airways. The conducting airways extend from the trachea to the terminal bronchioles and their chief function is to conduct air in and out of the lungs. The respiratory airways comprise of respiratory bronchioles, alveolar ducts and alveolar sacs. The respiratory airways are involved in gas
exchange where oxygen and carbon dioxide are exchanged between air and pulmonary capillaries(1, 2).

According to model proposed by Weibel et. al. there are 24 airway generations; trachea being generation 0 and alveolar sacs being generation 23. The airways show a dichotomous branching pattern such that the number of airways in one generation is double than that in the previous generation (3). Figure 1.1 shows the model of airway generation(4).

The diameter of airways decreases from 18 mm in the trachea to 0.4 mm in the alveoli while the total surface area increases from 2-3 m² in the conducting airways to >100m²(1). The conducting airways are mainly composed of ciliated columnar epithelial cells which are covered by mucus lining. The coordinated beating of cilia propels the mucus upwards toward the pharynx where it is either swallowed or expelled. This process is known as the
mucociliary escalator which helps in clearing out the inhaled particles that are trapped in the mucus. In contrast, the alveoli is covered by broad and extremely thin squamous cells known as the alveolar type I cells through which oxygen and carbon dioxide diffuse during gas exchange. Alveolar type II cells are progenitor cells for type I cells, are distributed in the corners of the alveolar sacs, and secrete alveolar surfactant which decreases the surface tension in the alveolar walls during inspiration(1, 4).

1.2. Particle deposition in lungs

Inhaled particles can be either solid or liquid and are usually deposited in large conducting airways comprising of oropharynx, trachea, and bronchi, smaller respiratory bronchioles or terminal bronchioles and alveoli (5). The deposition of particles in the respiratory tract depends mainly upon particulate factors particle size, shape, density, and particle surface properties and upon pathophysiological factors such as respiratory rates and breathing patterns (tidal volume, breathing frequency, and flow rate). Normal lung physiology and function can change during asthma, bronchitis or other diseases states and affects the pulmonary deposition pattern of particles (5-7). However, the majority of literatures address the influence of particulate factors on pulmonary deposition.

Specifically, the diameter of an inhaled particle that deposits in the airways is commonly referred to as the aerodynamic diameter. The aerodynamic diameter is defined as the diameter of the sphere with a unit density that has the same terminal settling velocity in still air as the particle in consideration(8). A generated aerosol is usually polydisperse collection of particles suspended in an airstream. The resulting particle size distribution of an inhaled aerosol is characterized by the mass median aerodynamic diameter (MMAD)(9). The size distribution of an aerosol is best represented by a log-normal
distribution and the median can be obtained from this distribution curve. When the curve is plotted with reference to the mass of deposited aerosol then the median size is defined as the mass median aerodynamic diameter. Parameters like the geometric standard deviation (GSD) and percentage fine particle fraction (% FPF) are also used to measure the aerosol properties. These parameters are discussed in detail in the later section. Inhaled particle deposition in the lungs occurs by three main mechanisms namely impaction, sedimentation and diffusion.

Particles suspended in inhaled air follow a tortuous path through the branching airways. Particle momentum along the airway trajectory potentially results in impaction on the airway surfaces every time as the airflow direction changes. Large sized aerosol particles and droplets which are flowing through a rapidly moving air stream tend to deposit on the walls of large airway bifurcations by inertial impaction. This force of inertial impaction mainly occurs in the oropharynx and the large airways where flow velocities are high and rapid changes in airflow direction occurs (10). Typically particles having MMAD values >5µm are mainly deposited in the oropharynx and the large airways by inertial impaction(5). If an inhaled particle avoids inertial impaction in the upper airways, gravitational sedimentation then becomes a more prominent force to promote deposition in the peripheral regions of lungs such as the respiratory bronchioles and the alveoli. Particle deposition by gravitational sedimentation in the lower portions of the lung can be promoted through patient factors such as breathing rates, tidal volumes, and breath holding (6, 10). Particles having MMAD values between 1-5µm optimize deposition in the smaller airways and alveoli(5). Submicron particles undergo Brownian Motion to a much more significant effect than larger sized particles with particles having MMAD
values ≤ 0.5 µm having substantial deposition in the alveoli where the airway diameter is small, air velocity is low and residence time is long(10). Additionally, particles having submicron aerodynamic sizes can be exhaled due to a failure to impact or settle in lung tissue. Figure 1.2 indicates that optimal particle deposition in the bronchoalveolar region requires that the particles be small enough to overcome inertial impaction in the upper airways and penetrate into the lower airways but large enough to avoid exhalation (5).

Figure 1.2: The effect of particle size on deposition of particles in the human respiratory tract following slow inhalation and a 5-second breath hold (4)

The ability to control particle deposition in the respiratory tract can be important in infectious lung diseases like pneumonia. In these types of infections, microorganisms multiply and divide in the bronchoalveolar region and require anti-infective agent presence at the site of infection. One proposed treatment option for pneumonias is the targeted delivery of aerosolized anti-infective agents to the deep lung with aerodynamic
diameters between 1-5 µm for optimal deposition of the agent to the bronchioalveolar region of the respiratory tract.

1.3. Next Generation Impactor and aerodynamic parameters

The particle size distribution is an important feature in determining the performance of an inhaled aerosol. While the particle shape and size distribution can be determined by microscopy, laser diffraction, light scattering techniques; the aerodynamic particle size distribution is typically determined by cascade impactors. The aerodynamic diameter has been strongly correlated with regional lung deposition and this is observed in Figure 1.2. The lung is a natural aerosol filter where large particles can be collected in the upper airways and smaller particles can deposit in the deep lungs. Cascade impaction is an *in vitro* method used to classify aerosol by size and is used to accesses the delivery efficiency of the inhaled products or formulations. The impactor instrument is used to obtain the size distribution of an aerosol produced by a specific formulation and device through analysis of the active pharmaceutical ingredient (API) in the sample. Unlike other techniques which use a representative sampling to determine the particle size and distribution, cascade impactors measure the size distribution on the entire drug sample which is delivered from a device. The impactor separates the aerosolized particles from a moving airstream on basis of inertial impaction and converts deposited masses into aerodynamic measures that correlate particle size, shape, and density into standardized aerodynamic descriptions of the aerosol. As previously described, the aerodynamic diameter is the defined as the diameter of a hypothetical spherical particle of unit density (i.e. $\rho_{\text{ae}} = 1.00 \text{ g/cm}^3$) that settles in air at the same falling velocity as the physical particle.
In most general cases for non-spherical particle the aerodynamic diameter \( d_{ae} \) is given by the equation 1.1(11).

\[
d_{ae} = d_p \sqrt[4]{\rho_p C_p / \rho_{ae} C_{ae}}
\]

Equation 1.1: Aerodynamic diameter of non-spherical particle

Where \( d_{ae} \) is the aerodynamic diameter of a particle

\( d_p \) is the diameter of spherical particle

\( \rho_p \) is the particle density

\( C_p \) is the Cunningham slip correction factor for particle of size \( d_p \)

\( X \) is the dynamic shape factor

\( \rho_{ae} \) is the unit density (i.e, 1.00 g/cm\(^3\))

\( C_{ae} \) is the Cunningham slip correction factor for particle of size \( d_{ae} \)

As previously mentioned, the cascade impacter works on the principle of inertial impaction. Inertia is the tendency of an object to resist any change in motion. Particles moving in an airflow stream possess an inherent inertia due to which the particle continues to move in the original air flow direction even when the direction of air flow changes. The particles which lose their inertia due to the friction between other particles or surrounding air molecules flow in the direction of altered air flow. Particles which lose their inertia are said to relax in the new air flow direction(12). In the cascade impactor,
collection plates are placed directly in the airflow path and provide a surface for particle impaction by causing airflow redirection. Large sized particle with high inertia will resist the change in direction of airflow and impact on the surface of collection plates. On the contrary, smaller sized particles which lose their inherent inertia and relax quickly into the new air flow direction, do not impact on the collection surface and continue to flow in the altered air flow direction. Additionally, cascade impactors have a series of nozzles with decreasing diameters through which the air flows. These decreasing diameter nozzles cause increased linear air flow velocity throughout the instrument and increases the particle inertia and subsequent particle deposition in the corresponding collection plates. The measured particle size distribution of an aerosol is then a function of the correlated masses of the API, impactor design and airflow rate employed. Impactor calibration is carried out at known air flow rates and linear velocities and assumes a standardized distance between the nozzles and the collection surface (12), (9).

The USP approved cascade impactor devices include the 8-Stage non-viable Andersen impactor, Marple-Miller impactor, multistage liquid impinger (MSLI), the Next Generation Impactor (NGI) are the various instruments which have been approved by the USP for pharmaceutical aerosol testing. Although pharmaceutical aerosols have been tested by various approved devices, the development and approval of the Next Generation Impactor (NGI) by a consortium of industrial and regulatory scientists have led to the adoption this new impactor for the majority of aerosol formulation and device formulation.

The NGI is a cascade impactor with seven stages (Stage 1-7) aligned horizontally in series with the final stage termed a micro-orifice collector (MOC). The different parts of
the NGI are depicted in Figure 1.3. Each stage and the MOC are composed of a nozzle affixed to the seal body and a removable impaction cup in the bottom frame with the lid that contains the inter-stage air flow passageways. Initially the NGI was developed and calibrated for flow rates from 30-100 L/min with pharmaceutical applications of dry powder inhaler and pressurized metered dose inhaler testing. The 50% efficiency cut-off diameters of the stages (D50 values) range between 0.24-11.7 μm, evenly spaced on a logarithmic scale. At those air flow rates, there are always at least five stages with D50 values between 0.5 μm and 6.5 μm for the optimal evaluation of inhalable particles. The European Pharmaceutical Aerosol Group (EPAG) calibrated the NGI at 15 L/min and confirmed its suitability for nebulizer testing. At this air flow rate, the NGI had cutoff diameters in the range of 14.1-0.98 μm, with the last five stages having cutoff diameters between 5.39 and 0.98 μm. These studies proved the suitability of the NGI for testing the inhaled dry powders and nebulized liquids with cut-off diameters of all the stages of the NGI at 60 L/min and 15 L/min provided in Table 1.1.
<table>
<thead>
<tr>
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<th>$D_{50}$ (µm)</th>
<th>60 L/min</th>
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<tr>
<td>1</td>
<td>8.06</td>
<td>14.1</td>
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<td>2</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>0.34</td>
<td>0.98</td>
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</tr>
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Table 1.1: $D_{50}$ values for NGI stages at air flow rates of 60 L/min and 15 L/min

Figure 1.3: Parts of the Next Generation Impactor

NGI calibration at different airflow rates allows for the determination of USP-defined aerosol aerodynamic parameters including the percentage fine particle fraction (% FPF),
mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD). The % FPF is the percentage of particles in fine particle range (1-5 μm) and calculated as the fraction of total drug deposited in stages which have cut-off diameter less than or equal to 5 μm(13),(14). The USP defines the total delivered drug mass from the inhaler device into the impactor as \( \sum A \). The total mass of drug deposited in the impactor stages which have cut-off diameter less than or equal to 5 μm is depicted as \( \sum B \) and is determined based on the cutoff diameters determined at different airflow rates through the NGI. The % FPF is then calculated as shown in Equation 1.2. For example, \( \sum A \) equal to the total drug mass deposited in the induction port, preseparator and stages of the impactor while \( \sum B \) is the sum of drug mass deposited from stage 2-7 for an air flow rate of 60 L/min and from stage 4-7 for an air flow rate of 15 L/min.

\[
% \text{ FPF} = \frac{\sum A}{\sum B} \times 100
\]

Equation 1.2: Calculation of percentage fine particle fraction

The aerodynamic particle size distribution of the aerosols is often referred to in terms of the MMAD and the GSD. Both these parameters are determined from the plot of the cumulative percentage of deposited mass in the stages versus the particle size as determined by the stated aerodynamic diameter \( D_{50} \) as shown in Figure 1.4. The particle size distribution usually conforms to log-normal frequency function. The MMAD is then defined as the particle size at 50% of the deposited cumulative mass so that 50% of the deposited mass is composed of larger particles and 50% of the deposited mass has smaller particles. The GSD is the measure of the breadth of the log-normal distribution and gives an indication if the aerosol particle size is monodisperse or polydisperse. The
GSD is derived by the square root of the particle size at the 84\textsuperscript{th} percentile (indicated as ‘X’ in Figure 1.4) to the particle size at 16\textsuperscript{th} percentile (indicated as ‘Y’ in Figure 1.4) as explained in Equation 1.3.

![Graph of cumulative percentage of mass less than the stated aerodynamic diameter versus aerodynamic diameter](image)

Figure 1.4: Plot of cumulative percentage of mass less than the stated aerodynamic diameter versus aerodynamic diameter

\[
GSD = \sqrt{\frac{\text{Size } X}{\text{Size } Y}}
\]

Equation 1.3: Determination of geometric standard deviation

1.4. Targeted lung delivery

The lungs have been a major route of drug administration since historic times to treat variety of diseases. Targeted delivery of drugs to the lungs is considered as the first line of therapy in treating localized disease such as asthma and chronic obstructive pulmonary disease (COPD) for many years. Recently targeted lung delivery is gaining popularity as a possible route for drug delivery in treatment of systemic diseases like diabetes(15). However, the main aim in delivering drugs to the lungs is to achieve precise dose
deposition past the oropharynx in the central or peripheral airways for the treatment of local conditions. The advancements in inhaler devices and particle engineering have promoted optimized drug deposition at the site of disease. The major advantages of targeted lung delivery are listed in Table 1.2. Targeted lung delivery helps to locally deliver high drug concentration directly to the site of disease and can minimize the risk of systemic side effects. For example, the systemic therapy of tobramycin is associated with risk of nephrotoxicity and ototoxicity while no toxicity has been reported in several well controlled trials following multiple courses of inhaled tobramycin (16). Ideally, a similar or superior therapeutic response can be achieved by targeting drugs to the lungs. For example, 2-4 mg of oral salbutamol is therapeutically equivalent to 100-200 μg of metered dose inhaler (MDI) of salbutamol (17). This can be in part to low concentrations of drug metabolizing enzymes present in the lungs. Drugs delivered through the pulmonary route by-pass first pass metabolism and often undergo less degree of metabolism than the drugs which are administered orally. Targeted pulmonary delivery of drugs helps to generate rapid clinical response as compared to systemic drug delivery(4). Ailments such as asthma can produce symptoms like bronchospasm within seconds with the targeted pulmonary delivery of bronchodilators providing rapid clinical response. However, there are few limitations involved with targeted lung delivery which are listed in Table 1.3. The lungs have a lower buffering capacity than the other delivery sites such as the gastrointestinal tract or blood. Thus only limited excipients which are biocompatible with the airways can be incorporated in the inhaled formulations. Lactose is the only excipient which is approved by the FDA for use in DPI (14). There is a huge inter and intra-subject variability in dose delivered. This variability is attributed to the
patient factors such as age, breathing pattern, inspiratory flow and degree of airway obstruction. The dexterity of patient in handling the inhaler devices also affects the dose delivered from the device. The current inhaler devices are inefficient where there is wasting of drug dose because a portion of dose is retained in the devices at the end of the treatment (18). Controlling the particle size and size distribution of the powder is the main challenge in formulating dry powder inhalers. Particle engineering techniques need to be employed to produce dry powders in the in range of 1-5μm. In case of nebulized aerosols, the nebulizers must be efficient in producing aerosol droplets in the desired particle size range.

<table>
<thead>
<tr>
<th>Advantages of targeted lung delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted delivery of potent drug dose to disease site</td>
</tr>
<tr>
<td>Minimal risk of systemic side effects</td>
</tr>
<tr>
<td>Similar or superior therapeutic effect at fraction of systemic dose</td>
</tr>
<tr>
<td>Bypass first pass metabolism in liver and poor GIT absorption</td>
</tr>
<tr>
<td>Rapid onset of action</td>
</tr>
</tbody>
</table>

Table 1.2: Advantages of targeted lung delivery

<table>
<thead>
<tr>
<th>Limitations of targeted lung delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompatibility of formulations with the airways</td>
</tr>
<tr>
<td>Inter and intra-subject variability in the dose delivered</td>
</tr>
<tr>
<td>Production of aerosols in narrow size range of 1-5 μm</td>
</tr>
<tr>
<td>Patient compliance is device dependent</td>
</tr>
</tbody>
</table>

Table 1.3: Limitations of targeted lung delivery
1.5. Inhalation devices

In case of targeted delivery of drugs to the lungs, the delivery system must be able to conveniently deliver optimum and reproducible respirable doses, efficiently and consistently produce aerosols with particle size range of 1-5μm, and maintain the chemical and physical stability of the formulation. Two of the main classifications of devices used for aerosolization of a drug formulation for inhalation purposes include nebulizers and dry powder inhalers (DPI). These device classes produce aerosols in different manners and have various advantages and disadvantages listed in Table 1.4.

1.5.1. Nebulizers

Nebulizers are widely used for delivering drugs to the respiratory tract mainly in case of patients in hospital or nonambulatory setting. There are three main types of nebulizers based on how the aerosol is produced: air jet nebulizers, ultrasonic nebulizers, and vibrating mesh nebulizer. The air jet nebulizers generate aerosol by atomization. A compressed gas is passed through a small orifice to generate low pressure at the outlet of the adjacent liquid feed tube. Thus as a result the drug solution is drawn up into the tube through a Venturi Effect to be atomized into small droplets in presence of the gas stream at the outlet of the orifice (19). Ultrasonic nebulizers use a piezoelectric crystal that vibrates at high frequency (1-3 MHz) to converts the liquid into aerosolized droplets with an MMAD of 3.0-3.6 μm. Vibrating mesh nebulizers uses a vibrating, perforated metal screen to produce droplets of uniform size dependent on the diameter of hole in the mesh. These devices aerosolize solutions or suspensions to a mouthpiece, ventilation mask, inline ventilation adapter, or tracheostomy (20). Air jet nebulizers tend to be time consuming for the dose to be administered, require an external air pump or air source,
produce an aerosol with a polydisperse particle size distribution, and are more prone to risk of microbial contamination if not cleaned properly. Additionally, these nebulizers have a substantial quantity of the dose that is retained in the device (also known as dead volume) and drug wastage to the environment during expiration with only 10% of the total drug dose actually reaching the lungs (19). Ultrasonic nebulizers tend to generate larger aerosol particles and have a higher mean output than air jet nebulizers. However, they are costly, require high maintenance and cleaning, and generate substantial heat and energy during aerosolization and possible drug degradation or formulation instability. Some of the dose tends to deposit on the device wall and the oral mucosa resulting in less drug dose reaching the lungs (21). Vibrating mesh nebulizers are portable, silent, fast and can aerosolize almost all the fluid resulting in significantly less drug loss as compared to the other two nebulizers. The temperature of the drug solution does not change during nebulization and thus there is less possibility of evaporative loss of the drug and also minimal chances of drug degradation or formulation instability. However, vibrating mesh nebulizers are very costly and are susceptible to clogging when used with suspension formulations. These devices also require regular cleaning (18).

When compared with other routes of administration, there are very few drug products that have been approved by the United States Food and Drug Administrative (FDA) for inhalation therapy. Even fewer antimicrobial agents have been approved for targeted delivery to the lungs. One example is inhaled tobramycin TOBI for management of cystic fibrosis patients with *Pseudomonas aeruginosa*. Therefore, clinicians have reported numerous studies involving the nebulization of intravenous antimicrobial formulations (22). The physical properties of aerosols produced by nebulization, including
nebulization rates and the aerosol particle size distributions can be significantly affected by the drug formulation. Formulation properties such as osmolality, pH, viscosity, ionic strength, surface tension and the presence of preservatives, co-solvents and stabilizers can dramatically affect aerosol generation, drug deposition, and patient adverse effects such as coughing, bronchoconstriction, and airway irritation or inflammation. For example, formulations with low pH or osmolalities <100 mOsm/kg or >1100 mOsm/kg have been reported to elicit cough and airway irritation and bronchospasm(21). A formulation viscosity >1.5cP has been associated with poor droplet aerosolization and low nebulizations rates (23).

1.5.2. Dry Powder Inhalers (DPI)

Dry powder inhalers are designed to aerosolize formulations that contain solid particles. These devices are often used to deliver API-containing formulations in which the drug is unstable in solutions or has a low aqueous solubility. The drug formulation is designed with a DPI device and to deliver either single doses or multiple doses. In the single dose device (e.g. Aerolizer, Rotahaler), the drug formulation is usually loaded into the device as a hard gelatin capsule or foil blister. In the multiple dose devices, the drug formulation is contained as multiple unit doses sealed in blister strips within the inhaler (e.g. Diskus) or as a bulk powder compact or reservoir within the device (e.g. Turbuhaler) (19). Currently examples of approved DPI devices include: a multi dose DPI of fluticasone and salmeterol, Advair for treatment of asthma and chronic obstructive pulmonary disease (COPD); a DPI of zanamivir, RELENZA for prophylaxis of influenza.

Often, drug formulations in DPI device include micronized drug is mixed with carrier particles to control drug particle aggregation and improve the powder flow and
aerosolization. Most DPI devices are breath actuated to passively aerosolize drug formulations during patient’s inspiratory air flow. Aerosolization of the activated drug dose occurs when air flows through the device and fluidizes the static powder bed to deliver powder to the patient’s airways. Thus the dispersion of the drug powder in air flow is directly dependent on the patient’s inspiratory flow rate(14). Dry powder inhaler formulations are usually high potency and contain predominantly the drug or are formulated with a carrier powder, often lactose. The inclusion of carrier particles seeks to optimize inter and intra-particulate interactions (governed by van der Waal’s forces, electrostatic attraction, and mechanical interaction) due to surface roughness and capillary forces due presence of adsorbed liquid layer to create a stabilized and easily aerosolized formulation. These particulate interactions must be overcome for drug particles to deaggregate and deposit in the airways. Physiochemical properties of the powder formulations, including particle size, shape, density, surface area, morphology, crystallinity, and moisture content must be evaluated to ensure efficient particle deposition of the drug in the lungs. The process by which dry powders are produced in a controlled fashion so as to obtain particle of optimum size, shape, morphology is known as particle engineering. The main objective of particle engineering for aerosolized powders is to produce particles with uniform size distribution, improved dispersibility, and enhanced drug formulation stability (8).

The DPI devices have many advantages such as they are portable and do not require the physical dexterity of other inhaler devices. However, the patient must be cognitively able to understand directions and be compliant with proper dose administration instructions. Also formulation products have assured stability is assured since the dry powders can be
prepared and packaged to decrease the rate of physical and chemical degradation. However, these passive devices require a high inspiratory airflow rate (approximately 60L/min) to adequately aerosolize the formulation which might be challenging in patients with impaired lung function such as asthmatic, pediatric, or geriatric patients.(20). Therefore, uniformity in drug dose can be problematic in these patients since the drug-carrier separation is inadequate because of the low inspiratory airflow(24). Thus active inhalers or breath assisted devices are under development where the inhaler synchronized formulation aerosolizes with the patient’s breath to help in aerosol generation. These active inhalers use pneumatics, impaction forces or vibratory energy to disperse the drug particles and are more independent of the patient’s inspiratory air flow in order to aerosolize the drug particles. These devices can be used in patients with impaired lung infection and in case of drugs with low therapeutic indices (14).

Various technique such as milling, spray drying, spray freeze drying, supercritical fluid technology are used to generate particles suitable for inhalation(25). Milling is a well-established technique for manufacturing dry powder for inhalation. In milling, the particle size reduction is mainly achieved by using pressure, friction, attrition, impact or shear. However the process of milling is time consuming, produces irregular dense particles, and is unsuitable for processing fragile biopharmaceuticals. Supercritical fluid technology utilizes supercritical fluids (e.g. carbon dioxide) which exist as compressed gases or liquids above their critical pressure and temperature. This technology is mainly based on supercritical antisolvent precipitation to produce pure and composite particles suitable for inhalation. Super critical fluid technology produces particles which have narrow size distribution and controlled morphology and the technique can also be used
for processing of heat liable biological materials. Spray drying is a process wherein liquid solution is sprayed in small droplets which are immediately dried by heated drying air. Spary freeze drying is an extension of spray drying where liquid droplets are sprayed into cryogenic solution like nitrogen or argon. Due to the low boiling point of the cryogenic liquid, the liquid droplets are frozen and are recovered by lyophilization. The spray drying and the spray freeze drying generally produce low-density particles suitable for inhalation. The next section mainly deals with the principle and process parameters involved in the process of spray drying.

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer</td>
<td>No specific inhalation technique or co-ordination required</td>
<td>Time consuming, bulky, non-portable and expensive</td>
</tr>
<tr>
<td></td>
<td>Aerosolizes solutions or suspensions</td>
<td>Contents can be easily contaminated</td>
</tr>
<tr>
<td></td>
<td>Large drug dose delivered</td>
<td>Poor delivery efficacy and high drug wastage</td>
</tr>
<tr>
<td></td>
<td>Suitable for infants, asthmatic patients and people who are sick or physically unable to use other devices.</td>
<td>Large variations between different models and operating conditions</td>
</tr>
<tr>
<td>Dry Powder Inhaler</td>
<td>No hand mouth co-ordination required as compared to metered dose inhalers</td>
<td>Deposition efficiency in the lungs dependent on patients inspiratory flow, not suitable for children &lt; 5years and asthmatic patients</td>
</tr>
<tr>
<td></td>
<td>Compact and portable</td>
<td>Dose lost if patient inadvertently exhales into the DP, potential problems in dose uniformity</td>
</tr>
<tr>
<td></td>
<td>Easy to use</td>
<td>Humidity can cause powders to aggregate and capsules to soften</td>
</tr>
<tr>
<td></td>
<td>The breath of patient is used to activate the device and deliver the dose.</td>
<td>Development and manufacturing process complex than nebulizers</td>
</tr>
</tbody>
</table>

Table 1.4: Advantages and disadvantages of nebulizers and dry powder inhalers
1.6. Spray Drying

Spray drying is a single step process which converts liquid into dry particles through evaporation of the solvent or dispersed phase (8). The technique of spray drying is widely used in food, chemical, cosmetic, and pharmaceutical applications. Pharmaceutical applications of spray drying include the processing of small drug molecules, proteins, peptides, and vaccines for oral administration, reconstitution and injection. Spray drying has also been reported for formulating dry powder inhalation of various drugs like tobramycin, amikacin, capreomycin, ciprofloxacin, salbutamol, budesonide; various proteins and peptides like insulin, albumin, deoxyribonuclease and various excipients like lactose, mannitol(8).

The principle of spray drying is based on the evaporation of solvent from liquid droplets to yield dry powder. The droplets are formed by spraying a liquid solution, suspension, or emulsion through a nozzle into a heated stream of drying gas, typically air. Once the liquid has evaporated, particles pass through a collection chamber for separation from the drying gas. A schematic diagram of a spray dryer with the associated processing parameters is depicted in Figure 1.4. The principle spray drying process operations include atomization, drying and separation.

The first operation is atomization, in which the liquid is pumped through a nozzle. Various nozzle types can be used and include a pressure nozzle, two fluid nozzle, rotary disk atomizer or an ultrasonic nozzle. These nozzle types impart different types of energy for the dispersion of the liquid feed into fine aerosol droplets. The droplet size produced through atomization is a primary determinant of the final particle size.
The second operation is drying in which atomized droplets are sprayed into the drying gas to promote liquid evaporation. The second operation of spray drying process is drying (26). When the droplets come in contact with the heated gas, evaporation of solvent takes place from the droplet surface due to high temperature of the heated gas. This results in formation of saturated vapor film at the droplet surface. Due to the high surface area of the droplets, a temperature and moisture gradient developed. There is an intense heat and mass transfer which results in efficient drying. The driving force in solvent removal is the difference in the partial water vapor pressure between the solid surface and the environment. The drying continues until the driving force decreases to zero when there is no residual solvent left in the solid particles. Thus the final moisture level in the product is determined by the humidity of drying conditions, nature of the product and the temperature of the heated gas (27). Evaporation of the solvent leads to cooling of droplet eventually less thermal load on the droplet. During the drying process, the temperature at the droplet surface is equal to the wet bulb temperature. The wet bulb temperature is the lowest temperature which can be reached due to evaporation of water. The drying time usually extends from 100 milliseconds to a few seconds before the product is removed from the drying chamber. Thus there is very little likelihood of the product getting damaged by heat (27, 28).

Evaporation is promoted as the air and droplet remain in contact by three different flow currents and which eventually affect the properties of dry powder. In the co-current flow the droplets are sprayed in the same direction as that of the drying air while counter-current flow has droplets that are sprayed in the opposite direction as that of the drying air. Combining both of the method uses the advantages of both the co-current and
counter-current flow to promote optimal contact with drying air. Counter-current flow is used mainly for thermo-stable products due to the longer contact times with the heated drying gas. If the drying gas is exhausted to the atmosphere, the system is an open cycle. If the heated gas is to be recycled and reused, an inert gas such as nitrogen is employed and such a system is known as a closed cycle system. Closed cycle systems are mainly used when flammable gases or solvents are used or when toxic or oxidation-prone products are spray dried.

The third operation of spray drying is separation of the dried product that occurs in the separation device, usually a cyclone. The cyclone chamber separates the product by using centrifugal force where the air is set in rotational motion and the particles are directed to the walls of device and get separated from the air stream. The separated dry powder gets collected in a collection cup which is attached to the bottom of the cyclone chamber.

Within these three operations, four spray drying process parameters of inlet temperature, sample feed rate, drying gas flow rate, and spray gas flow rate can be altered to optimize the spray drying process. These parameters mainly affect the particle size, yield, and moisture content of the final powder. Table 1.5 shows the effect of relationship between process parameters and final product properties.

The inlet temperature is the temperature of drying air when the drying air comes in contact with feed solution prior to atomization. The inlet temperature determines the amount of solvent that is removed per unit time. The outlet temperature is the resultant temperature due to heat exchange between the drying air and the liquid droplets and cannot be directly regulated. In practice, the outlet temperature is the highest temperature which the product can reach. The drying gas flow rate is the volume of the drying air
supplied to the system per unit time and determines the drying level of the product and the degree of product separation in the cyclone. The spray gas flow rate determines the pressure of drying gas flowing through the nozzle to atomize the liquid feed into small liquid droplets. The feed rate determines the volume of liquid feed supplied through the nozzle by peristaltic pump in unit time (29).

Figure 1.5: Process diagram of Buchi mini spray dryer B-290
<table>
<thead>
<tr>
<th>Spray parameters</th>
<th>Inlet Temperature</th>
<th>Drying gas flow rate</th>
<th>Spray gas flow rate</th>
<th>Feed rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Properties</td>
<td>Outlet temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No effect</td>
<td></td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moisture content</td>
<td></td>
<td></td>
<td>No effect</td>
</tr>
<tr>
<td></td>
<td>No effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yield</td>
<td></td>
<td>No effect</td>
<td>Depends on application</td>
</tr>
</tbody>
</table>

Table 1.5: Relationship between process parameters and properties of final product

↑: Increases  ↓: Decreases

Pulmonary drug delivery is being currently used or is considered as an option for treatment of chronic obstructive pulmonary disease (COPD), cystic fibrosis, lung infections like pneumonia, lung cancer and also for delivering vaccines and in gene therapy. The next section mainly deals with pneumonia especially MRSA acquired pneumonia and its treatment.

1.7. Pneumonia

1.7.1. Definition
Pneumonia is defined as an acute lower respiratory tract infection mainly caused by microorganisms like bacteria, virus or fungi and usually occurs in respiratory bronchioles and alveoli(30, 31). Pathological changes in pneumonia include retention of fluid and immune cells due to an increased infiltration of polymorphonuclear leukocytes in within the lung which cause an increase in lung weight and induration. Clinical manifestations of pneumonia include new opacities on chest radiography with the patient exhibiting at least two of the following signs and symptoms: cough, sputum, pleuritic chest pain, oral temperature above 38°C, difficulty breathing, or lung tissue consolidation.(30-32)

1.7.2. Epidemiology:

In the United States, pneumonia is sixth most common cause of death with more than 80,000 people dying each year of bacterial pneumonia(33). Pneumonia occurs in all age groups, although very young and elderly individuals are more prone to the infection. Risk factors for developing pneumonia include human immunodeficiency virus (HIV) infection, diabetes, renal failure, cancers, malnutrition, cigarette smoking and treatment with immunosuppressive drugs. Patients with underlying lung diseases like chronic obstructive pulmonary disease (COPD), cystic fibrosis, lung cancer and congestive heart failure as also those on mechanical ventilation are at an increased risk of developing pneumonia. (31)

1.7.3. Pathogenesis and etiology:

Microorganisms enter the respiratory tract by: inhalation wherein microbes, carried in small droplets are inhaled into the tracheobronchial tree; aspiration of oropharyngeal secretions containing pathogens into the lungs, or extrapulmonary infiltration of pathogenic organisms from a bloodstream infection (33).
In case of healthy individuals with optimal pulmonary defense mechanisms, most pathogenic organisms can be cleared from the lungs before they can cause pneumonia. However, even healthy individuals with unimpaired defense mechanisms can develop viral pneumonia.

Individuals with impaired lung defenses can suffer from life threatening pneumonia caused by pathogenic organisms. Factors which lead to impaired lung defenses are viral upper respiratory tract infections, ethanol abuse, cigarette smoking, heart failure and chronic obstructive pulmonary disease. Viral infections suppress the antibacterial activity of alveolar macrophages and impair mucociliary clearance resulting in bacterial pneumonia(34). Mucociliary transport is suppressed by ethanol and there is obstruction of bronchus by tumor and mucus which result in impaired clearance of aspirated bacteria. Individuals suffering from acquired immunodeficiency syndrome, certain malignancies and those using cytotoxic drugs and/or corticosteroids have compromised lung defense(33).

1.7.4. Classification of pneumonia:

Pneumonia is classified into two types: Community Acquired Pneumonia (CAP) and Hospital Acquired Pneumonia (HAP). This classification is based primarily on the setting in which pneumonia is developed.

1.7.4.1. Community acquired pneumonia (CAP):

Community acquired pneumonia (CAP) refers to the lung infection that develops in a community setting and which is not acquired in a hospital setting. In almost 50% of patients the cause of CAP is never identified. *Streptococcus pneumonia* is the most
common bacterial pathogen which causes CAP. Other pathogens which can cause CAP include *Haemophilus influenza, Mycoplasma pneumonia*, influenza A, and newer pathogens such as *Legionella* species and *Chlamydia pneumonia*. Methicillin resistant *Staphylococcus aureus* which is a main causative pathogen of hospital acquired pneumonia has emerged as a prevalent pathogen in CAP as well. Patients who are suffering from comorbid illness like chronic obstructive pulmonary disease (COPD), congestive heart failure, asthma, diabetes or who have received recent treatment with antibiotics or those who have been admitted to nursing home in the recent past are at an increased risk of developing CAP(33). Patients suffering from CAP have been divided into four subcategories. The first group consists of patients with no coexisting illness or other risk factors, who have not been on antibiotic treatment for past three months and those who do not require hospitalization. The second group consists of patients with coexisting illness or other risk factors, who have been on antibiotic treatment for past 3 months but who can still be treated without hospitalization. The third and fourth group consists of patients which suffer from severe CAP. The third group of patients requires hospitalization whereas the fourth groups of patients suffer from the most severe CAP and require admission in the intensive care unit (33, 35, 36). The causative pathogens and initial antibiotic therapy for the treatment of the above four subcategories of CAP is
summarized in Table 1.6 (Weinberger et al.).

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Common Organisms</th>
<th>Other Miscellaneous Organisms</th>
<th>Initial Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outpatient, no cardiac pulmonary disease or other modifying risk factors</td>
<td>S. pneumoniae M. pneumoniae P. aeruginosa H. influenzae (in smokers)</td>
<td>Legionella M. tuberculosis Endemic fungi</td>
<td>Advanced generation macrolide (e.g., azithromycin or clarithromycin) or Doxycycline</td>
</tr>
<tr>
<td>Outpatient, with cardiovascular disease and/or other modifying factors</td>
<td>S. pneumoniae M. pneumoniae H. influenzae Aerobic gram-negative bacilli Respiratory viruses Anaerobes C. pneumoniae</td>
<td>M. catarrhalis Legionella M. tuberculosis Endemic fungi</td>
<td>Oral quinolone (with activity against pseudomonus) or β-Lactam plus macrolide</td>
</tr>
<tr>
<td>Hospitalized</td>
<td>S. pneumoniae H. influenzae Pseudomonas aeruginosa (including P aeruginosa) Aerobic gram-negative bacilli (legionella) C. pneumoniae Respiratory viruses</td>
<td>M. tuberculosis</td>
<td>Intravenous (IV) β-lactam plus IV or oral macrolide or doxycycline or IV quinolone</td>
</tr>
<tr>
<td>Hospitalized, severe pneumonia</td>
<td>S. pneumoniae Legionella H. influenzae M. catarrhalis Aerobic gram-negative bacilli M. tuberculosis Respiratory viruses S. aureus</td>
<td>M. tuberculosis Endemic fungi</td>
<td>IV β-lactam plus either IV macrolide (azithromycin) or IV quinolone</td>
</tr>
</tbody>
</table>

Table 1.6: Causative organisms and initial therapy in CAP(33)

1.7.4.2. Nosocomial pneumonia

Nosocomial pneumonia is defined as the pneumonia that was not present or incubating at the time of admission to the hospital. Hospital acquired pneumonia (HAP) is the pneumonia which occurs more than forty-eight hours after hospitalization and without any signs of infection at the time of hospital admission. Ventilator associated pneumonia (VAP) is a subset of HAP and it results from extended mechanical ventilation. VAP normally occurs within 48-72 hours of mechanical ventilation (37). It is necessary to classify nosocomial pneumonia as distinct from community acquired pneumonia, since patients with nosocomial pneumonia are susceptible to different and potentially more
virulent spectrum of organisms. HAP accounts for almost 15% of all hospital acquired infections (38), while VAP occurs in almost 10-20% of intubated patients (39). Hospitalized patients have increased susceptibility to HAP mainly due to the colonization of the oropharynx by virulent pathogenic organisms and the micro-aspiration of these colonized pathogens to the lower respiratory tract. Supine positioning has also demonstrated to increase the risk of HAP in hospitalized patients (38). In case of VAP, the use of endotracheal tubing mitigates the aspiration of the colonized pathogens in the lower respiratory tract. The intubation process also impairs the protective defense mechanisms like the cough reflex and the mucociliary clearance of secretions, thus preventing the aspirated colonized secretion from being expelled out. In addition, instrumentation of the nose increases the incidence of sinusitis thereby increasing the risk of aspiration (39). Patient related factors like age, severity of underlying illness like diabetes, heart disease, chronic obstructive pulmonary disease, prolonged hospitalization, malnutrition, coma or use of sedating agents and prior antibiotic exposure can increase the risk of HAP and VAP by adversely affecting the host immunity. Risk factors related to infection control include poor hand washing practice, inappropriate use of gloves and contaminated respiratory devices and equipment. All the above risk factors result in the weakening of the host defense. Thus due to the compromised host immunity, the pathogens which enter the lower respiratory tract colonize the airways and cause pneumonia (40). HAP is classified as into two categories early onset and late onset HAP. Early onset HAP is the pneumonia which arises less than 5 days into a hospital course and the late onset HAP is the pneumonia which arises 5 days or later into a hospital course. These two categories are further subdivided into patients with prior antibiotic
exposure and patients without prior antibiotic exposure. The causative pathogens in case of early onset HAP in patients with no prior antibiotic exposure mainly include Enterobacteriaceae, Haemophilus influenzae, Streptococcus pneumoniae, and methicillin-sensitive Staphylococcus aureus. Patients who are recently exposed to antibiotics are infected with aforementioned pathogens including non-lactose fermenting gram-negative bacteria. The causative pathogens in case of late-onset HAP in patients with no prior antibiotic exposure is caused by pathogens listed above. On some occasions the patients are infected with gram-negative bacilli which are resistant to first-generation cephalosporins. In case of late-onset HAP with prior antibiotic exposure, almost 40% of the patients are infected with multidrug-resistant pathogens (MDR) such as Pseudomonas aeruginosa, Acinetobacter baumannii, and methicillin-resistant Staphylococcus aureus (MRSA) (38). VAP is also classified as early-onset and late-onset pneumonia. Early-onset VAP occur within 48-72 hours of ventilator support is frequently caused by antibiotic-sensitive bacteria, including methicillin-sensitive Staphylococcus aureus (MSSA), Haemophilus influenzae, and Streptococcus pneumoniae. Late-onset VAP occurs more than 72 hours after start of mechanical ventilation and is caused by antibiotic resistant pathogens including MRSA, Pseudomonas aeruginosa, Acinetobacter spp., and Enterobacter spp (41). In immunocompetent patients, VAP could be caused by anaerobic bacteria, Legionella pneumohila, and Candida spp. In case of patients with early onset of pneumonia and no additional risk factors, the initial therapy should be limited to either third generation cephalosporin, fluoroquinolones, penicillins with gram-negative coverage but no antipseudomonal activity or carbapenems with gram-negative coverage but no antipseudomonal activity. In case of patients with late-onset of pneumonia and risk
of multi-drug resistant pathogen, the initial therapy should be a combination of antipseudomonal beta-lactam agents plus aminoglycosides or antipseudomonal fluoroquinolones. Vancomycin or linezolid and antifungal agents need to be incorporated if the patient is infected with MRSA and fungal pathogens (37). The causative pathogens and initial therapy for early-onset and late-onset HAP and VAP pneumonia is enlisted Table 1.7.
<table>
<thead>
<tr>
<th>Category</th>
<th>Potential pathogens</th>
<th>Initial empiric therapy</th>
</tr>
</thead>
</table>
| Early onset pneumonia with no known risk factors of MDR pathogens      | ➢ *Streptococcus pneumonia*  
➢ *Haemophilus influenza*  
➢ Methicillin-sensitive *Staphylococcus aureus*  
➢ Antibiotic-sensitive enteric gram-negative bacilli  
  • *Escherichia coli*  
  • *Klebsiella pneumoniae*  
  • *Enterobacter* species  
  • *Proteus* species  
  • *Serratia marcescens* | Ceftriaxone  
OR  
Levofoxacin, moxifloxacin or, ciprofloxacin  
OR  
Ampicillin/sulbactam  
OR  
Ertapenem |
| Late onset pneumonia with known risk factors of MDR pathogens          | ➢ Gram-negative bacilli  
  • *Klebsiella pneumoniae*  
  • *Enterobacter* species  
  • *Pseudomonas aeruginosa*  
➢ *Acinetobacter* species  
➢ ESBL-producing *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus* species | Cefepime or ceftazidime  
OR  
Doripenem, imipenem, or meropenem  
OR  
Piperacillin/tazobactam PLUS  
Ciprofloxacin or levofloxacin  
OR  
Amikacin, gentamicin, or tobramycin |
|                                                                        | ➢ Methicillin-resistant *Staphylococcus aureus*                                                       | Vancomycin or linezolid                                                                   |
|                                                                        | ➢ *Legionella pneumophila*                                                                           | Azithromycin and fluconazole                                                              |

Table 1.7: Potential pathogens in early and late-onset HAP and VAP and initial empiric therapy(37)
1.7.5. **Methicillin Resistant *Staphylococcus aureus* (MRSA) pneumonia**

According to the National Nosocomial Infection Surveillance, *Staphylococcus aureus* is the most common pathogen causing nosocomial pneumonia accounting for 17% of isolates. There is an increasing emergence of antibiotic-resistant strains with more than 60% *S. aureus* being methicillin resistant(42). Nosocomial pneumonia is associated with increased morbidity, mortality and health care costs(43). In a survey carried by Kollef et. al. in 4543 patients with culture-positive pneumonia in 59 US hospitals, MRSA was identified as the potential causative pathogen in 8.9% of community acquired pneumonia (CAP), 26.5% of healthcare-associated pneumonia (HCAP), 22.9% of hospital-acquired pneumonia (HAP), and 14.6% of ventilator-associated pneumonia (VAP) cases (43).

Specific risk factors that increase susceptibility to pneumonia caused by MRSA include intubation or prolonged mechanical ventilation, increased duration of stay in the Intensive Care Unit, and previous exposure to broad spectrum antibacterial treatment (44). Elderly patients and those suffering from significant underlying diseases are also more prone to infection with hospital acquired MRSA pneumonia (45).

SCCmec is a genetic element of *S. aureus* associated with emergence of methicillin resistant strains. SCC stands for staphylococcal chromosomal cassette and mec is the gene encoding methicillin resistance. The mec encodes PBP2A, a penicillin binding protein which has intrinsic reduced affinity for methicillin and all β-lactams. The mec gene is present in *S. aureus* for it to show resistance to penicillin and cephalosporin. There are five types of SCCmec from I to V(46). The strains of MRSA which cause HCAP, HAP and VAP are labeled as hospital-acquired MRSA (HA-MRSA) and contain SCCmec types I-III while the MRSA strains that cause CAP are referred to as community-acquired MRSA (CA-MRSA) and contain SCCmec type IV. The clinical
manifestation of HA-MRSA pneumonia is generally indistinguishable from the pneumonia caused by other pathogens. Staphylococcal pneumonia is a necrotizing infection which causes rapid tissue destruction and cavitation and can lead to local complications like abscesses and pleural emphysema. HA-MRSA pneumonia is associated with mortality rate of 55.5% despite early and appropriate therapy\(^{(45, 47)}\). CA-MRSA strains usually cause pneumonia in young, previously healthy patients with a preceding influenza like illness. CAP is characterized by severe respiratory symptoms, hemoptysis, high fever, leukopenia, very high C-reactive protein level (>400 g/L), hypotension, and a chest x-ray showing multi lobular cavitating alveolar infiltrates\(^{(45)}\).

The American Thoracic Society has issued guidelines for management of adults with HAP, VAP and HCAP. According to the guidelines, when a pathogen is identified a pathogen specific therapy should be used. According to the ATS guidelines, intravenous vancomycin is one of the recommended regiments for the treatment of MRSA acquired nosocomial pneumonia and is considered to be the drug of choice in treating MRSA infections. Specifically, the recommend dosing guidelines for intravenous vancomycin are 15mg/kg every 12 hour with optimal serum trough levels of vancomycin in range of 15-20 μg/mL\(^{(37)}\). However, treatment failure with vancomycin occurs mainly due to its poor penetration in the alveolar lining fluid. Researchers have tried to correlate the blood plasma vancomycin concentration and epithelial lining fluid concentrations. Two studies have been reported where the level of vancomycin in the lung epithelial lining fluid (ELF) is in the range of 11-18 % of the blood plasma concentration, indicating the poor penetration of vancomycin in the alveolar lining fluid \(^{(48, 49)}\).

1.7.6. Vancomycin hydrochloride
1.7.6.1. **Drug type**

Vancomycin hydrochloride is the salt form of a tricyclic glycopeptide antibiotic that is produced by *Streptomyces orientalis* (Fam. Streptomycetaceae). According to USP, vancomycin hydrochloride has a potency equivalent to not less than 900 μg of vancomycin per mg, calculated on the anhydrous basis (50). Current vancomycin use is for the treatment of severe systemic infections caused by gram-positive bacteria in patients who could not receive or who had failed to respond to penicillin and cephalosporins or for the treatment of gram-positive bacterial infections that were resistant to β-lactams and other anti-infective agents. On April 15th 1986, FDA approved vancomycin hydrochloride for oral delivery for treatment of staphylococcal enterocolitis and antibiotic-associated pseudomembranous colitis caused by *Clostridium difficile*.

1.7.6.2. **Chemical name and formulae**

The vancomycin molecule is a very large glycopeptide (Figure 1.6) with a very long chemical IUPAC name: \((S_a) - (3S,6R,7R,22R,23S,26S,36R,38aR)-44\-\[2-O-(3-Amino-2,3,6-trideoxy - 3 - C - methyl - α - L - lyxo - hexopyranosyl) - β - D - glucopyranosyl]oxy] - 3 - (carbamoylmethyl) - 10,19 - dichloro - 2,3,4,5,6,7,23,24,25,26,36,37,38,38a - tetradecahydro - 7,22,28,30,32 - pentahydroxy - 6 -(2R) - 4 - methyl - 2 - (methylamino)valeramido] - 2,5,24,38,39 - pentaoxo - 22H - 8,11:18,21 - dietheno - 23,36 - (iminomethano) - 13,16:31,35 - dimetheno - 1H,16H - [1,6,9]oxadiazacyclocexadecino[4,5 - m][10,2,16]benzoxadiazacyclotetracosine - 26 - carboxylic acid, monohydrochloride. It is commonly referred to as vancomycin with proprietary names of Vancocin®, Lyphocin®, and VancocinHClPulvules®.

The molecular empirical formula for vancomycin hydrochloride is \(C_{66}H_{75}Cl_{2}N_{9}O_{24}\cdot HCl\).
Vancomycin hydrochloride has a molecular weight of 1485.73 g/mol.

1.7.6.3. Physicochemical properties

Vancomycin hydrochloride is a white to yellowish-white solid powder. It does not have a sharp melting point but decomposes at a temperature in excess of 200°C. Vancomycin hydrochloride is soluble in water (>100 mg/mL), moderately soluble in dilute ethanol, and insoluble in higher alcohols, acetone and ethers. The numerous functional groups impart multiple protonation states to vancomycin with reported pKa values of 2.18, 7.75, 8.89, 9.59, 10.4, and 12.0.

1.7.6.4. Pharmacology

Vancomycin is slowly bactericidal in nature and by disrupting cell wall synthesis in gram-positive bacteria through inhibition of peptidoglycan biosynthesis. Vancomycin is a glycopeptide antibiotic which inhibits the polymerization or transglycosylase reaction by binding to the D-alanyl-D-alanine terminus of the cell wall precursor unit attached to its lipid carrier depicted as LCP-NAM-NAG in Figure 1.6 and blocks the linkage of
precursor unit to the glycopeptide polymer depicted as \((\text{NAM-NAG})_n\) which are located within the bacterial cell wall (51). Bacterial vancomycin resistance mainly occurs due to expression of enzymes that modify the cell wall precursor so that it no longer binds to vancomycin.

![Figure 1.7: Inhibition of bacterial cell wall synthesis by vancomycin](image)

Vancomycin has numerous approved indications for various infections that are susceptible to its use, resistant to other antibiotics, and in patient populations that are not able to use other antibacterial agents. Of note, vancomycin is indicated for initial therapy when methicillin-resistant staphylococci are suspected, but after susceptibility data are available, therapy should be adjusted accordingly. Additionally, vancomycin efficacy has been reported in lower respiratory tract infections. However, it should be used only to treat or prevent infections that are proven or strongly suspected to be caused by susceptible bacteria. This precaution helps to reduce the emergence of vancomycin resistant bacteria (FDA labels).

Vancomycin is contraindicated in patients with known hypersensitivity to the antibiotic.
1.7.6.5. Adverse effects and toxicity

Systemic vancomycin administration should be via intravenous (IV) infusions to avoid tissue irritation and necrosis associated with intramuscular (IM) injections with special precautions taken to avoid extravasation. However, pain and thrombophlebitis often occur following IV administration in many patients, and occasionally may be severe. Red man syndrome is a condition associated with rapid intravenous infusion of vancomycin and is characterized by erythematous reactions, flushing, tachycardia and hypotension. Red man syndrome is not an allergic reaction but it is related to induced mast cells histamine release and is more common with rapid infusion of high dose vancomycin over a short period (52). Ototoxicity and nephrotoxicity are serious side effects associated with parenteral vancomycin therapy. Ototoxicity may be transient or permanent, with tinnitus sometimes reported as a precursor before the onset of deafness, and necessitates discontinuance of the drug. However, deafness may develop despite cessation of vancomycin therapy (53). Ototoxicity has been reported to occur when vancomycin plasma concentration are between 60-100 μg/mL. Nephrotoxicity has been associated with high serum vancomycin levels and is a particular concern in patients that receive concomitant aminoglycoside therapy (51). Vancomycin induced nephrotoxicity has been associated with transient elevations in serum creatinine concentration and the presence of hyaline and granular casts and albumin in the urine (53).

1.7.6.6. Problems associated with intravenous vancomycin therapy

Vancomycin is considered as a gold standard therapy in treatment of MRSA acquired pneumonia. According to the American Thoracic Society guidelines, a 15mg/kg dose of vancomycin should be administered intravenously every 12 hours and the plasma trough
concentration (minimum concentration $C_{\text{min}}$) of vancomycin should be 15-20 μg/ml (37). However clinical failure is often reported especially for the treatment of nosocomial MRSA pneumonia with reported treatment success rates of 35-68% (Table 1.8). Important factors affect the clinical outcome of intravenous vancomycin therapy in treatment of nosocomial pneumonia including microbial susceptibility, lung permeability, serum vancomycin levels and systemic side effects.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Percentage clinical success rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fagon et al (54)</td>
<td>58.3%</td>
</tr>
<tr>
<td>Rubinstein et al (55)</td>
<td>68.1%</td>
</tr>
<tr>
<td>Wunderink et al (56)</td>
<td>35.5%</td>
</tr>
<tr>
<td>DeRyke et al (57)</td>
<td>58.3%</td>
</tr>
</tbody>
</table>

Table 1.8: Clinical success rates of vancomycin therapy for treatment of MRSA acquired nosocomial pneumonia

1.7.6.6.1. *In vitro* susceptibility

The minimum inhibitory concentration (MIC) of vancomycin for MRSA is found to be in the range of 0.5-2.0 μg/mL (58). In 2006, the Clinical and Laboratory Standards Institute lowered the vancomycin susceptibility breakpoint for *S. aureus* from 4 μg/mL to 2 μg/mL (59) because researchers reported that there is a statistically significant association with more frequent vancomycin treatment failures as the MIC of vancomycin increases (60). Other researchers reported that regardless of the MIC values, an initial response rate of 74 % was achieved if the target trough concentration was ≥15 μg/mL. They also observed that in spite of achieving trough concentrations at the end of 72 hours of therapy, the
response rates were significantly lower for patients infected with strains having an MIC of 2 μg/mL as compared to those having MIC of 1 μg/mL or less (85% vs 62%) (61).

1.7.6.6.2. Area under inhibitory curve

The area under the inhibitory curve (AUIC) is the ratio area under the concentration curve compared with the MIC (AUC/MIC). Studies have proposed that the AUIC is an important predictor of clinical success of vancomycin. Moise-Border and colleagues carried two retrospective analyses to determine the relationship between AUIC and the clinical response during treatment of hospitalized patients suffering from MRSA infection with vancomycin. In the first study, there were 70 patients infected with methicillin susceptible and resistant *S. aureus* and suffering from lower respiratory tract infection. It was seen that patients with predicted AUIC value of ≥ 345 had better clinical outcome (78% success rate) than the patients with predicted AUIC value of < 345 (24% success rate). The second study was carried on 108 patients infected with methicillin susceptible and resistant *S. aureus* and suffering from lower respiratory tract infection. This study demonstrated that clinical and microbiological response to vancomycin therapy was superior in patients with AUIC values ≥ 400 (46)(62). These studies helped to determine the relationship between vancomycin dosage, serum concentration, MIC and the antibacterial efficacy.

1.7.6.6.3. Lung penetration

The process of vancomycin diffusion from the pulmonary capillaries into the lung tissue occurs by passive transport and is affected by protein binding, drug molecular weight, lipid solubility, capillary density, capillary and membrane permeability, tissue inflammation, membrane surface area, oncotic and osmotic gradients (63). Cruciani et al
determined vancomycin lung penetration in 30 patients undergoing lung resection. Each patient received a single 1g dose of vancomycin by IV infusion for 1 hour with samples collected for up to 12 hours after infusion. At the end of 12 hours the ratio of vancomycin concentrations in the lung to tissue concentrations was 0.41. They also reported that the administration of single 1g dose of vancomycin by IV infusion for 1 hour was not able to maintain the recommended serum trough concentration of 15-20 μg/mL (64). Other researchers’ investigated the relationship between lung penetration and lung inflammation while using the albumin concentration in the lung epithelial lining fluid (ELF) as a marker of lung inflammation. Vancomycin penetration in ELF was determined by the ratio of vancomycin concentration in ELF to that in plasma. This ratio was significantly higher in patients with high ELF albumin concentrations as compared to patients with normal ELF albumin concentrations (24.6% versus 14%) and reported that vancomycin penetration is higher in patients with inflammation, mainly due to increased membrane permeability. Additionally, vancomycin concentrations in ELF were only 18 % of those in plasma (48).

The American Thoracic Society guidelines has advocated that the vancomycin dosing of 15mg/kg every 12 hours should achieve serum trough concentrations of 15-20 μg/mL so that the vancomycin concentration is at least 2 μg/mL in the epithelial lining fluid for the drug to show its efficacy against MRSA. Vancomycin should be able to penetrate deep in the alveoli which are the site of MRSA infection. The vancomycin should be in a free state (unbound form) for it to penetrate the lung. Vancomycin protein binding in the serum is approximately 55 %. When vancomycin is administered intravenously, it has to cross two membrane barriers before it reaches the site of bacterial infection. The first
membrane is the nonfenestrated capillary endothelial membrane which limits the drug penetration. The second membrane is alveolar epithelial membrane which forms a formidable barrier due to the presence of tight intracellular junctions. Thus vancomycin is temporarily located in the interstitial space. However the interstitial fluid is actively cleared by lymphatic clearance. The protein binding and rapid clearance from the interstitial space results in poor penetration of vancomycin in the alveoli (63).

1.7.6.6.4. Nephrotoxicity and red man syndrome

Historically, toxicities due to vancomycin administration were reported due to impurities in the manufacturing process. It was seen that patients who were administered vancomycin for longer duration (greater than 21 days), who had vancomycin trough serum concentration (greater than 10 mg/l) and who were concurrently treated with aminoglycosides were at a greater risk of developing nephrotoxicity, although the incidence of vancomycin nephrotoxicity is low (65). Hidayat et al compared the incidence of nephrotoxicity to high (≥15 μg/mL) and low (< 15 μg/mL) vancomycin trough concentrations. It was seen that nephrotoxicity associated with vancomycin administration was 12% in case of patients with high trough concentration and 0 % in case of patients with low trough concentration(61). In other study, Rybak et al demonstrated that the patients who were administered vancomycin and gentamicin in combination demonstrated higher incidence of nephrotoxicity when compared with patients receiving vancomycin alone (22 % versus 5 %) (65). Jeffers et al suggested that in patients suffering from MRSA acquired HCAP; the incidence of nephrotoxicity is significantly higher in patients who are on aggressive vancomycin dosing to attain a maximum vancomycin trough concentration ≥15 μg/mL(66).
Red man syndrome is an infusion related hypersensitivity reaction peculiar to vancomycin. It typically causes pruritus, an erythematous rash that involves the face, neck, and upper torso and less frequently hypotension and angioedema. Vancomycin causes degranulation of mast cells and basophil resulting in the release of histamine. The histamine release is dependent to amount and rate of vancomycin infusion. Studies have shown that more severe reactions occur in younger patients younger than 40 years, particularly in children(67). Polk et al observed that 82% of volunteers receiving 1g vancomycin infusion every 12 hours suffered from red man syndrome. It was also seen that all the volunteers receiving 500mg vancomycin infusion every 6 hours did not suffer from red man syndrome. It was seen that plasma histamine concentrations measured every 10 minutes after each infusion increased in subjects who were administered 1g dose and there was only a slight change in histamine levels after administration of 500-mg dose(68). Healy et al carried out a randomized, double-blind, two-way crossover trial to determine effect of 1g vancomycin infusion over 1 hour and 2 hours. 80% of h volunteers who received infusion over 1 hour experienced the red man syndrome as compared to only 30% of volunteers who received infusion over 2 hours. The peak concentration of histamine in plasma and the total histamine released in plasma was greater in case of 1 hour infusion (69). Thus it can be concluded from these studies that red man syndrome occurs due to vancomycin infusion rate-dependent increase in plasma histamine concentration.

1.7.7. Hypothesis and specific aims

A logical approach to overcome the limited effectiveness of intravenous vancomycin therapy is to target the delivery of vancomycin to site of infection in the lungs. Targeted
lung delivery will help to achieve high vancomycin concentration at the site of infection and minimize the risk of systemic side effects. Preclinical aerosolization of vancomycin was performed in an isolated rat lung after pulmonary and systemic delivery. It was observed that pulmonary delivery of vancomycin led to high drug levels in bronchoalveolar lavage fluids (BALF) (> 100 μg/mL) and the lung tissue (> 50 μg/g) as compared to systemic administration where the vancomycin levels were undetectable in BALF and very low drug levels were obtained in the lung tissue (70). Thus this study demonstrated that pulmonary delivery of vancomycin could be used as an alternative route of delivery.

There are few clinical cases which have reported of nebulized intravenous vancomycin formulation for lung infections (Table 1.9), with no published reports dealing with development, characterization and testing of aerosolized vancomycin hydrochloride. Additionally, there is a paucity of controlled clinical trials to support the efficacy of aerosolized vancomycin for treatment of pneumonia. The formulations aspects and delivery techniques for administering aerosolized vancomycin are poorly studied, unstandardized, and necessitate further study.
<table>
<thead>
<tr>
<th>Clinical Studies</th>
<th>Dose of vancomycin</th>
<th>Diluent</th>
<th>Frequency of dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hayes et al (71)</td>
<td>Standard intravenous VH (250mg)</td>
<td>5mL sterile saline</td>
<td>Twice daily for 6 months</td>
</tr>
<tr>
<td>Maiz et al (72)</td>
<td>Preservative free intravenous vancomycin chlorhydrate (250mg)</td>
<td>4mL sterile water</td>
<td>Twice daily for 17 months</td>
</tr>
<tr>
<td>Gradon et al (73)</td>
<td>500mg vancomycin</td>
<td>3mL sterile water pH (3.6)</td>
<td>1ml dose every 6 hours via face mask</td>
</tr>
<tr>
<td>Weathers et al (74)</td>
<td>Intravenous solution of vancomycin (500mg)</td>
<td>5mL 0.95% NaCl injection pH (3.52)</td>
<td>40mg delivered 3 times daily via nebulizer</td>
</tr>
</tbody>
</table>

Table 1.9: Clinical reports describing the use of nebulized vancomycin to treat MRSA lung infections

The main goal of the present research was to develop inhaled vancomycin hydrochloride formulations which could be potentially used to target the lungs and serve as an alternative to the present intravenous therapy for treatment of MRSA acquired pneumonia. The first aim was to prepare, test and evaluate the aerosol characteristics of inhaled formulations of vancomycin hydrochloride. The aerosol properties of two
different inhaled formulations of vancomycin hydrochloride were tested and compared so as to obtain the formulation with better *in vitro* deposition efficiency. The formulations were also tested and compared for anti-bacterial efficacy to determine the effect of processing parameters on the drug stability and to obtain formulations with better anti-bacterial efficacy. Nebulized solutions and spray dried powder inhalations of vancomycin hydrochloride were prepared and characterized. The *in vitro* deposition efficiency of these formulations was tested using the Next Generation Impactor and the anti-bacterial activity was determined using Methicillin resistant *Staphylococcus aureus* bacterial strain. The second aim was to develop a proof of concept for an *in vitro* impactor model which could be used as a two-in-one equipment to test the aerosol properties and anti-bacterial efficacy. This model could be possibly used as a tool for screening aerosolized antibiotic formulations. Thus the principal hypothesis of this research was:

**Dry powder inhalation of vancomycin hydrochloride has better *in vitro* deposition efficiency and equivalent therapeutic efficacy compared to a nebulized solution of vancomycin hydrochloride**

In order to prove the above hypothesis the research study had two specific aims:

**Specific aim A**: Comparison of Nebulized solution and Dry powder inhalation of Vancomycin hydrochloride for *in vitro* deposition efficiency and therapeutic efficacy

**Specific Aim B**: Developing a proof of concept for an *in vitro* model using Next generation impactor as a potential screening tool for aerosolized antibiotics
Chapter 2

Specific Aim ‘A’:

Comparison of \emph{in vitro} deposition efficiency and therapeutic efficacy of nebulized solution and dry powder inhalation of vancomycin hydrochloride
2.1. Analytical method development and validation using UPLC

2.1.1. Introduction
Vancomycin is a highly polar tricyclic glycopeptide antibiotic. Vancomycin is available commercially as the hydrochloride salt. It has a molecular weight of 1485.71 Daltons. It is mainly used in the treatment of respiratory tract infections such as pneumonia caused due to Methicillin Resistant *Staphylococcus aureus* (MRSA) bacteria.

2.1.2. Materials
Vancomycin hydrochloride (USP grade) was obtained from PCCA (Houston, Texas). Potassium dihydrogen phosphate, phosphoric acid and acetonitrile was obtained from Fischer Scientific (Pittsburgh, PA).

Liquid chromatography was performed using an Acquity UPLC BEH C\textsubscript{18} 1.7 μm 2.1 x 100 mm (Waters Corporation, Milford, MA) and a Acquity UPLC system (Waters Corporation, Milford, MA) consisting of a sample manager, column manager, photodiode array detector (PDA) and binary solvent manager. Data was analyzed using Empower Pro software from Waters Corporation (Milford, MA).

2.1.3. Methods
2.1.3.1. Chromatographic conditions
A stability indicating chromatographic method for vancomycin using Ultra Pressure Liquid Chromatography (UPLC) has been reported and consists of a gradient with pH 3.2 buffered aqueous phase and 5-15% acetonitrile with a Waters BEH C18 column at a 0.4mL/min flow rate to elute a vancomycin peak within approximately 4 minutes. Vancomycin was detected using UV detection at 230nm (75). This method was modified by changing the gradient conditions to reduce the run time and ensure elution of any
residual impurities. The chromatographic column used was an Acquity UPLC BEH C\textsubscript{18} 1.7 μm 2.1 x 100 mm column. The column was maintained at 35°C throughout the analysis. The aqueous mobile phase (A) consisted of 95:5 (v/v) 5mM KH\textsubscript{2}PO\textsubscript{4} in water and acetonitrile. The apparent pH of the above solution was adjusted to 3.0 with phosphoric acid using UltraBASIC pH meter (Denver Instruments, Bohemia, NY). The organic mobile phase (B) consisted of 100% (v/v) acetonitrile. A gradient was used at a flow rate of 0.4 mL/minutes: 95% A to 85% for 2.0 minutes, 85% A to 5% A for 0.3 minutes and 5% A to 95% for 0.2 minutes. Each injection volume was 5 μL. The total run time for each run was 2.5 minutes. The effluents were analyzed at 230nm using PDA detector.

2.1.3.2. Preparation and validation of standard solutions
Solutions of vancomycin were made by dissolving 4.0 mg of vancomycin hydrochloride in 10mL of aqueous mobile to give a stock solution of 400μg/mL. Dilutions were prepared from this stock solution.

A standard curve was obtained by injecting solutions of vancomycin to correlate UV absorbance with known concentrations. Unknown vancomycin concentrations were then extrapolated from this regression equation. The chromatographic method was validated for its specificity, linearity, accuracy and precision as per USP guidelines for validation of analytical procedures.

2.1.4. Results and Discussion

2.1.4.1. Specificity
Specificity, in relation to chromatography, is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components (76). Figure 2.1 is a representative UV chromatogram without vancomycin present and has no sharp absorbance peaks. In contrast, Figure 2.2 is a representative chromatogram obtained after injection of 0.39 μg/mL vancomycin. These figures demonstrate that UV absorbance is sufficient to identify vancomycin and that chromatographic conditions do not interfere with the peak of interest. The retention time of vancomycin hydrochloride was found to be 1.186 minutes. This method has specificity for vancomycin even at a low drug concentration.

![Figure 2.1: A representative chromatogram obtained after injection of aqueous mobile phase without vancomycin hydrochloride](image)

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2.1.4.2. Linearity

The linearity of an analytical method is defined as the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range (76). In order to determine the linearity of an analytical method, a standard curve was plotted using a series of known concentration of the analyte and the respective peak areas obtained after injecting the analyte samples in the chromatographic system. The correlation coefficient of the plotted absorbance versus concentration curve was 0.9999 over a concentration range of 0.09-100μg/mL (Figure 2.3). The equation governing this relationship was found to be $y = 25359x - 5774.5$, where the vancomycin concentration is “x” in units of μg/mL and “y” is the peak area obtained using the Empower software from the analyte chromatogram.
2.1.4.3. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value (76). The accuracy of an analytical method correlates to the true value of a measurement and should be determined using at least nine samples with at least three concentrations that cover the entire range of standard curve are repeated a minimum of three times. The percentage accuracy is calculated as:

\[
\% \text{ Accuracy} = \frac{\text{Experimental Concentration}}{\text{Theoretical Concentration}} \times 100
\]

The tested vancomycin concentrations were 3.125 μg/mL, 12.5 μg/mL, and 50 μg/mL.

Table 2.1 lists the percentage accuracy values for the three quality control samples and
were found to be > 90% with a percentage relative standard deviation (% RSD) of less than 5%, which was within acceptable limits.

<table>
<thead>
<tr>
<th>Theoretical concentration (μg/mL)</th>
<th>Experimental concentration (μg/mL)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.125</td>
<td>3.12 ± 0.03</td>
<td>99.77 ± 1.01</td>
</tr>
<tr>
<td>12.5</td>
<td>11.80 ± 0.10</td>
<td>94.37 ± 0.80</td>
</tr>
<tr>
<td>50</td>
<td>48.27 ± 0.87</td>
<td>96.55 ± 1.74</td>
</tr>
</tbody>
</table>

Table 2.1: The percentage accuracy values for three quality control samples

2.1.4.4. Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample (76). The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Measurements of precision should be determined using at least nine samples with at least three concentrations that cover the entire range of standard curve are repeated a minimum of three times. The UPLC method for quantitative determination of vancomycin was tested for within and day to day precision. For determining the within day precision of the UPLC method, vancomycin solutions were prepared and injected three times on the same day. For determining the day to day precision of the UPLC method, vancomycin solutions were freshly prepared and injected on three different days over a period of thirty days. The % RSD values for within day and day to day precision
were calculated and were found to be <10% and <15% respectively, which was within the acceptable limits. The average value of the slope of standard curve on each day was found to be 24387.33 with % RSD of 12.31. Table 2.2 represents the within day and day to day precision values for UPLC analysis of vancomycin.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Within day</th>
<th>Day to day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean peak area</td>
<td>% RSD</td>
</tr>
<tr>
<td>0.09</td>
<td>2398.00</td>
<td>3.92</td>
</tr>
<tr>
<td>0.19</td>
<td>4633.33</td>
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</tr>
<tr>
<td>0.39</td>
<td>9470.00</td>
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<td>0.78</td>
<td>18546.00</td>
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</tr>
<tr>
<td>1.5</td>
<td>37506.67</td>
<td>2.26</td>
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<td>25</td>
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<tr>
<td>100</td>
<td>2599073.33</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 2.2: Within day and day to day precision values for UPLC analysis of vancomycin hydrochloride

2.1.5. Conclusion

A rapid analytical method for quantification of vancomycin was developed and validated using Ultra Pressure Liquid Chromatography. The analytical method was validated using
standard validation protocols as specified by the USP and was specific, linear over the concentrations tested, accurate, and precise.

2.2. *In vitro* deposition efficiency of nebulized solution of vancomycin hydrochloride

2.2.1. Introduction

The targeted delivery of antibiotics to the lungs helps to achieve high drug concentration at the site of infection with minimal risk of systemic side effects as compared to the systemic delivery of antibiotics. Traditionally antibiotics have been delivered to the lungs as solutions or suspensions using nebulizers which produce fine liquid droplets that are inhaled through a mask or mouthpiece. Nebulizers do not require a special inhalation technique but are inhaled using normal breathing patterns. Nebulizers can deliver large drug doses and are used for patients in all age groups. However, nebulizers tend to be bulky, are nonportable and can require long times to take a dose. There is also substantial variation involved in delivery efficiency between different models of nebulizers (19). Since few antibiotics have been formulated specially for aerosol delivery, intravenous formulations have been used as nebulized solutions for inhalation. The physical properties of intravenous antibiotic formulations like osmolality, pH, surface tension, viscosity, presence of preservatives must be evaluated and optimized before using the solutions for inhalation to minimize adverse effects like cough, airway irritation and bronchoconstriction.

2.2.2. Materials

Vancomycin hydrochloride (USP grade) was obtained from PCCA (Houston, TX). 0.9% sodium chloride solution (normal saline) was obtained from Braun Medical Inc. (Irvine,
CA). An Aeroneb Pro nebulizer (Aerogen Ltd, Galway, Ireland) and Next Generation Pharmaceutical Impactor (NGI) (MSP Corporation, Shoreview, MN) were used. Sigmacote (silicone solution in heptane) was obtained from Sigma Aldrich (St. Louis, MO).

2.2.3. Methods

Vancomycin hydrochloride solution (VH solution) was prepared by dissolving 40mg of vancomycin hydrochloride in 10mL of normal saline. 5mL of VH solution was used for nebulization and the remaining 5mL was used to determine the solution properties. The osmolality of VH solution was determined using micro osmometer (Precision Systems Inc, Natick, MA). The micro osmometer was calibrated using 100mOsm/kg and 500 mOsm/kg solutions. 50μL of VH solution was pipetted in 1.5mL conical vials and the osmolality was determined in triplicates. The pH of VH solution was determined by using the UltraBASIC pH meter (Denver Instruments, Bohemia, NY). 2 mL of VH solution was pipetted in glass vials and the pH was measured in triplicates.

The nebulization of drug solution was carried out using the NGI at an airflow rate of 15 L/min as verified using a TSI 4000 series airflow meter (TSI, MN, USA). An external terminal filter to capture fine particles was placed below the micro orifice collector (MOC) since the MOC becomes ineffective in collecting fine particles at air flow rate of 15L/min. The collection cups of the NGI were coated with Sigmacote to minimize or prevent particle bounce and/or reentrainment during impaction as described by Kamiya et. al.(77). Briefly, Sigmacote was applied to all the collection cups and the heptane was allowed to evaporate before the collection cups were assembled in the NGI. The effective
cut off diameters (D50 values) for stages 1-7 of the NGI at flow rate of 15 L/min were calculated as 14.1, 8.61, 5.39, 3.30, 2.08, 1.36 and 0.98 μm respectively (78). The nebulizer neck was placed in line with the induction port in such a way that all the aerosolized droplets exit the nebulizer neck and enter the induction port. The induction port was used to complete the NGI assembly as described in USP.

After the impactor was assembled, 5ml of VH solution was nebulized using the Aeroneb Pro nebulizer for approximately 8 minutes and was performed in triplicate. Drug was recovered by washing nebulizer and NGI components with aqueous mobile phase and analyzing the injected washings in the UPLC. The percentage of total drug recovered is determined as follows:

\[
% \text{ Dose Recovered} = \frac{\text{Amount of drug recovered from nebulizer and NGI parts}}{\text{Amount of drug aerosolized}} \times 100
\]

The amount of drug deposited in the induction port and the NGI is defined as the emitted dose (ED). The percentage fine particle fraction (% FPF), mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were the aerodynamic parameters which were calculated as specified in the USP (9). The % FPF \( \leq 5\mu m \) was calculated by interpolation to the cumulative percentage undersize at the impactor cut off diameter of 5μm (calculated from stage 4 to MOC) and represents the percentage of particles which have size of \( \leq 5\mu m \) (79). The MMAD and GSD were calculated from the plot of cumulative percentage of mass less than the stated aerodynamic diameter versus
the aerodynamic diameter as specified in the USP. The in vitro deposition efficiency of nebulized VH solution was determined from the aerodynamic parameters.

2.2.4. Results and Discussion

It is observed that nebulized drug solutions having osmolality values in the range of 150-1200 mOsm/kg and pH in range of 2.6-10 are generally well tolerated by patients with minimal risk of coughing and bronchoconstriction (80). The osmolality and the pH of VH solution was found to be 291.67 ± 0.58 mOsm/kg and 4.26 ± 0.01 respectively and was within the specified limits for nebulized drug solution.

Only 33.15 ± 1.18 % of the drug dose recovered from only the impactor was deposited in the final five stages which had D50 values ≤ 5μm. Figure 2.4 gives a graphical representation of the percentage of total dose deposited in each part of the nebulizer and impactor. The % FPF ≤5μm of the nebulized VH solution was found to be 34.01 ± 1.12 %. The MMAD and the GSD of the nebulized liquid droplets was found to be 8.89 ± 0.39μm and 2.29 ± 0.10 respectively.

It was seen that more than 90 % of the nebulized drug was recovered from the nebulizer device and the impactor. The therapeutic effect of drug is correlated to the mass of drug reaching the site of action; the particle size distribution of aerosol is expressed in terms of mass. The central tendency of distribution is determined by the mass median aerodynamic diameter (MMAD). The log-normal distribution around the MMAD is expressed in terms of geometric standard deviation (GSD) (81). The nebulized VH solution exhibited a MMAD of approximately 9 μm which was greater than the respirable size range of 1-5 μm. More than 50% of drug was deposited in the initial stages of NGI
and correspond to the upper respiratory region. Only 34% of the drug was deposited in the lower respiratory region which is the site of MRSA infection. The nebulization of VH solution resulted in formation of heterodisperse aerosol. This observation is indicated by the GSD value which was greater than 2.

2.2.5. Conclusion

The osmolality and pH of vancomycin hydrochloride solution were within the range specified for administration of aerosolized solutions to the lungs. Heterodisperse aerosols were formed with a high MMAD which resulted in low % FPF ≤5μm. Thus the nebulized vancomycin hydrochloride solution showed poor in vitro deposition efficiency.

Figure 2.4: The percentage of total dose deposited in the nebulizer and impactor
2.3. Preparation and characterization of spray dried powders for inhalation

2.3.1. Materials and Methods

2.3.1.1. Preparation of spray dried (SD) powders

Spray dried powders were produced using a low drug concentration 50mL of 5 mg/mL VH in water and high drug concentration 10mL of 50mg/mL VH in water. These solutions were then spray dried using a laboratory-scale mini spray dryer B-290 and inert loop B-295 (BÜCHI Labortechnik AG, Flawil, Switzerland) to obtain high-potency carrier free dry powders. The spray drying parameters are summarized in Table 2.3. Briefly, the aspirator delivered the drying gas at a flow rate of 100% (35 m³/hr) and influences the maximize degree of powder separation in the cyclone. Compressed nitrogen gas was circulated through the spray dryer so as to displace the oxygen from the system. Once the oxygen level in the spray dryer was less than 6%, the inlet temperature was set to 150° C which is the drying temperature at which the solvent is removed from the feed solution. The outlet temperature was in range of 68-80° C and is in practice the highest temperature at which the product can be heated. The spray gas flow rate was varied from 30mm to 60mm (357 to 742 L/hr) to influence the degree of atomization of the liquid feed into droplets. The drug solutions were delivered to the spray drier at a feed rate of 10% (3mL/min).
<table>
<thead>
<tr>
<th>Spray drying parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inlet temperature</td>
<td>150º C</td>
</tr>
<tr>
<td>Outlet temperature</td>
<td>68-80º C</td>
</tr>
<tr>
<td>% Aspiration</td>
<td>100 %</td>
</tr>
<tr>
<td>% Feed rate</td>
<td>10 %</td>
</tr>
<tr>
<td>Spray flow rate</td>
<td>30-60 mm</td>
</tr>
<tr>
<td>Feed concentration</td>
<td>5 mg/mL and 50 mg/mL</td>
</tr>
</tbody>
</table>

Table 2.3: Spray drying parameters

The spray dried (SD) powders obtained were collected and stored in a desiccator at room temperature for additional analysis.

2.3.1.2. Characterization of spray dried (SD) powders

2.3.1.2.1. Karl Fischer titrimetry

Karl Fischer titrimetry (Mettler DL18 Karl Fischer titrator, NJ, USA) was used to determine the moisture content in the SD and commercial vancomycin hydrochloride powders. The percentage moisture content in the powders was reported. The experiment was carried in triplicates.

2.3.1.2.2. Thermogravimetric analysis (TGA)

TGA analysis was carried for commercial vancomycin hydrochloride powder using thermogravimetric analyzer (Shimadzu TGA-50, Kyoto, Japan). Approximately 5mg of powder was weighed in aluminum pans and heated from 25º C(room temperature) to
150°C at rate of 10°C/min under nitrogen purge. This experiment was carried in triplicates.

2.3.1.2.3. Differential Scanning Calorimetry (DSC)

The thermograms of the SD and commercial vancomycin hydrochloride powder were obtained using Differential Scanning Calorimeter (Shimadzu DSC-60, Kyoto, Japan). Approximately 5 mg of the powders were weighed into aluminum pans which were heated from 25°C (room temperature) to 300°C at rate of 10°C/min under nitrogen purge.

2.3.1.2.4. Particle size analysis

The particle size of SD and commercial powder was determined using Malvern Mastersizer Hydro 2000S (Malvern, UK) which works on the principle of laser diffraction. Vancomycin hydrochloride is moderately soluble in dilute ethanol, insoluble in higher alcohols like isopropanol. Sufficient amount of sample was dispersed in isopropanol at 500rpm agitation and 20 % sonication for 3 minutes to obtain obscuration between 5-10 %. Three sets of measurements were repeated for three times for each sample. The D (0.5) is the median volume diameter at which 50% of sample is smaller and 50 % of sample is larger. The D (0.9) is the median volume diameter at which 90% of sample is smaller and 10 % of sample is larger. The D (0.1) is the median volume diameter at which 10% of sample is smaller and 90 % of sample is larger. D (0.1) and D (0.9) are mainly used to determine the range of particle size distribution. The D [4, 3] is the volume mean diameter which is the average diameter based on unit volume of particle. The D (0.5), D (0.1), D (0.9) and D [4, 3] were determined in micrometers.

2.3.1.2.5. Scanning Electron Microscopy (SEM)
The surface morphology of SD and commercial powders was determined using scanning electron microscopy. The samples were mounted on stubs which were coated with gold-palladium alloy and the examined using Scanning Electron Microscope (Quanta 200 by FEI) at an acceleration voltage of 25.0 kV.

2.3.1.2.6. Testing the *in vitro* deposition efficiency of dry powder inhalation of vancomycin hydrochloride

The aerosolization of the dry powder was carried out using the NGI at an airflow rate of 60 L/min. The NGI was assembled and operated as previously described but at an airflow rate of 60 L/min. The effective cut off diameters (D50 values) for stages 1-7 of the NGI at flow rate of 60 L/min were calculated as 8.06, 4.46, 2.82, 1.66, 0.94, 0.55 and 0.34 μm respectively (9). A custom made mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port. Sylgard® 184 silicone elastomer kit (Dow Corning, MI, USA) was used to prepare the mouthpiece adapter.

The mouthpiece adapter is attached to the induction port in such a manner that the mouthpiece of the inhaler is placed along the horizontal axis of the induction port. 20mg of VH commercial and SD powders were filled by hand in size 3 hard gelatin capsules (PCCA, TX, USA). The actual dose filled in the capsules was verified by weighing empty capsules and drug filled capsules. After the impactor was assembled, the drug filled capsule was placed in the Aerolizer DPI device attached to the NGI by way of the custom adapter. The NGI was operated for four seconds to draw 4L of air from the inhaler through the inhaler and aerosolize the powder. The deposited drug was recovered as previously described. The percentage of total drug recovered is determined as follows:
The in vitro deposition efficiency of the aerosolized VH powder was determined from the % FPF, MMAD and GSD. The % FPF ≤5μm was calculated by interpolation to the cumulative percentage undersize at the impactor cut off diameter of 5μm (calculated from stage 2 to MOC) (79). Thus % FPF ≤5μm is the percentage of particles which have size of ≤ 5μm. The MMAD and GSD were calculated from the plot of cumulative percentage of mass less than the stated aerodynamic diameter versus the aerodynamic diameter as specified in the USP.

2.3.1.2.7. Statistical analysis

Statistical analysis was carried out using the t-test: Two samples assuming equal variance from the data analysis tool of Microsoft Excel 2010.

2.3.2. Results and discussion

The spray drying process yielded eight different powders which were sprayed at different concentration and different spray flow rates. Four powders were obtained by spraying low concentration solution (5 mg/mL) at spray flow rates of 30, 40, 50 and 60 mm. Similarly, four powders were obtained by spraying high concentration solution (50 mg/mL) at spray flow rates of 30, 40, 50 and 60 mm.

2.3.2.1. Moisture Content
The inlet temperature is the temperature of the drying air at the moment of first contact with the feed. As the sprayed droplets come in contact with the heated drying air, evaporation takes place from the saturated vapor film which is formed at the surface of the droplet. This intense heat and mass transfer results in efficient drying (26). Incomplete removal of water from the powder can result in local drug dissolution and recrystallization resulting in solid bridge formation and producing irreversible aggregation of drug particles which can adversely affect the aerosol generation and lung deposition (14). The moisture content of commercial vancomycin hydrochloride powder was determined using the Karl Fischer titrimetry to be 10.73 ± 0.37 %. The moisture content of spray dried (SD) powders ranged from 7.47 % to 11.46 % (Table 2.4) and was similar to or less than the moisture content of commercial powder. The inlet temperature employed is the temperature of drying air which determines its ability to dry the liquid feed and also determine the final solvent content in the product (29). Thus the inlet temperature of 150 °C used in the spray drying process was efficient in removing water from the feed solution to yield dry powders for inhalation.

In case of the powders sprayed at a specified spray flow rates (40, 50 and 60 mm) at low (5 mg/mL) and high (50 mg/mL) concentrations, there was no significant difference (p>0.05) seen in the residual moisture content of these SD powders. Thus it can be concluded that the concentration of feed solution does not have any effect on the moisture content of vancomycin hydrochloride SD powders.
2.3.2.2. Thermal Analysis

The DSC profile of commercial vancomycin hydrochloride shows a broad endothermic event from 60-150º C (Figure 2.5) and has been discussed in the literature to be associated with dehydration of loosely bound water (82). The TGA analysis of commercial vancomycin hydrochloride showed a 9.66 ± 0.62 % weight loss when heated from room temperature to 150ºC and corresponds to the moisture content in the commercial vancomycin hydrochloride as determined by Karl Fischer titrimetry. Therefore the endothermic event from 60-150ºC for vancomycin hydrochloride is attributed to dehydration. Figures 2.5 and 2.6 depicts the DSC endotherms of commercial and SD vancomycin powders and also shows a broad endothermic dehydration event from 60-150º C which is similar to the commercial powder endotherm. The thermogram of commercial vancomycin hydrochloride shows an endotherm at around 210º C which could be attributed to the charring of drug that was confirmed by thermal microscopy. There is no change seen in the endotherms of powders sprayed at 5mg/mL and 50 mg/mL and at different spray flow rates and the endotherm of commercial vancomycin

<table>
<thead>
<tr>
<th>Spray flow rates (mm)</th>
<th>% Moisture content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low concentration</td>
</tr>
<tr>
<td></td>
<td>(5 mg/mL)</td>
</tr>
<tr>
<td>30</td>
<td>10.21 ± 0.15</td>
</tr>
<tr>
<td>40</td>
<td>10.27 ± 0.98</td>
</tr>
<tr>
<td>50</td>
<td>7.47 ± 0.53</td>
</tr>
<tr>
<td>60</td>
<td>11.46 ± 0.46</td>
</tr>
</tbody>
</table>

Table 2.4: Percentage moisture content of SD powders
hydrochloride. The chromatograms obtained after analyzing the spray dried powders using the UPLC were compared with chromatogram obtained after running commercial vancomycin on UPLC. The SD powders exhibited similar retention peaks as that of the commercial vancomycin. These findings indicate that vancomycin hydrochloride does not undergo any degradation at the spray drying temperature and also there is no change in the physical state of vancomycin hydrochloride after spray drying.

Figure 2.5: DSC endotherms of SD powders (5 mg/mL) and commercial powder
2.3.2.3. Particle size analysis

Table 2.5 summarizes the key size parameters, including the $D_{0.5}$, $D_{0.1}$, $D_{0.9}$ and $D_{[4, 3]}$ of each SD powder. The range of particle size is depicted by $D_{0.1}$ and $D_{0.9}$ values. The $D_{0.5}$ and $D_{[4, 3]}$ values of SD powders decreased as the spray flow rate increased. The higher the amount of atomizing air better is the atomization of liquid into smaller droplets and subsequently smaller is the size of product particles (29).

Compressed nitrogen gas is simultaneously fed through the nozzle along with the liquid feed so as to atomize the liquid feed into droplets. Elversson et al studied the effect of atomizing air flow rate on droplet size of aqueous lactose solution. It was seen that as the atomizing air flow rate was increased there was a decrease in the droplet size and eventually a decrease in the particle size (83). Atomization occurs because high frictional forces are generated over the liquid surface which causes the liquid to disintegrate into
droplets (29). It is been reported that frictional forces can cause conformation changes in biomolecules and could lead to protein denaturation (84). However, Stahl et al also demonstrated that the increasing the atomizing flow rate decreases the particle size of spray dried insulin without degrading the insulin (85). Thus the particle size of the final product is influenced by the spray flow rate which controls the amount of compressed gas flowing through the nozzle.

An increase in the concentration of feed solution increases the particle size of the spray dried particles. Particle size of spray dried trehalose and insulin particles have been reported to increase with an increase in feed concentration (86, 87). It is also seen that at all the spray flow rates the D [4,3] values increased as the concentration of feed solution increased from 5mg/mL to 50mg/mL. A liquid solution is sprayed in to small droplets which are instantly dried to yield dry particles in the spray dryer. When the atomizing air flow rate is kept constant, both the high and low concentration feed solutions will produce similar sized droplets. The amount of solid drug particles in each droplet depends on the concentration of the feed solution. So a solution sprayed at high concentration will produce droplets which have more solid content and hence result in formation of large sized dried particles (26). Assuming that single droplet dries to give single particle, the higher amount of drug per droplet would result in larger sized particles (86). A critical concentration is needed in the outer layer of the drying droplet to produce a dry particle. The drying process takes place by evaporation of the solvent from the outside of atomized droplet and concentration of dissolved solid increases until a critical concentration is reached. The droplet with higher solid content will reach the critical
concentration faster because amount of solvent to be evaporated is less and hence produce large sized particles as compared to droplets with lower solid content (83, 86).

The particle size data for commercial powder is presented in Table 2.6 and differs substantially from processed SD powders with a D (0.5) value of 35.23±2.10μm. It is seen that the almost all the SD powders had a narrow particle size range from 0.1-10 μm as compared to the commercial powder which had a broad particle size range from 3.0-1400 μm.

The SD powders were composed of a consistent population of small fine sized particles in the range of 0.1 to 1 μm for each set of processing conditions with variable population of larger particles. The fine sized particles possess high surface energy. These particles are stabilized by weak Van der waal’s forces by forming loose agglomerates which can be easily dispersed on application of energy. Hence these loose agglomerates could be easily dispersed during the particle size analysis where stirring and sonication was employed. The SEM images of SD powders showed the presence of these loose agglomerates which were broken down to their primary size during particle size analysis. Figures 2.7 depict the particle size distribution of representative powders which were sprayed at 5 and 50 mg/mL at 50 mm spray flow rate. The spraying of low concentrated solution leads to formation of droplets with low solid content and thus smaller fine sized particles. It is also seen that the dried particles can attach to a partially dried particle (86). There is an increased cohesiveness between the partially dried particles and completely dried particles which results in formation of strong solid bridges due to capillary forces resulting in formation of large sized particles (88). The powder obtained after spraying low concentration solution (5mg/mL) exhibited bimodal particle size distribution. The
bimodal distribution can be attributed to the presence of fine sized particle (< 1 μm) and large sized particle (1-5 μm). On spraying of high concentrated solution, the droplets contain a high solid content which eventually dry to give large sized particles. In addition due to increased solid content in the feed solution, there is a tendency of the droplets to coalesce and result in formation of large sized droplet which finally give large sized dry particle (83). Due to nonlaminar gas flow through the drying chamber, the sprayed droplets are dispersed in different air streams and are subjected to different local air temperature. Thus each droplet loses the solvent under different conditions and may result in formation of specific type of dried particle (29). The non-uniform solvent evaporation can generate build pressure in the drying particle and the particle can burst or explode into small fine particles(89). Thus spray drying high concentration solution can led to formation of large and fine sized particles. The powder obtained after spraying high concentration solution (50 mg/mL) exhibited unimodal particle size distribution in form of two distinct peaks where the larger peak was attributed to presence of large sized particles and the small peak was due to presence of fine sized particles.
<table>
<thead>
<tr>
<th>Spray flow rates (mm)</th>
<th>Concentration</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(5 mg/mL)</td>
<td>(50 mg/mL)</td>
</tr>
<tr>
<td></td>
<td>D(0.5) (μm)</td>
<td>D(0.5) (μm)</td>
</tr>
<tr>
<td></td>
<td>Range[D(0.1)-D(0.9)] (μm)</td>
<td>Range[D(0.1)-D(0.9)] (μm)</td>
</tr>
<tr>
<td></td>
<td>D [4,3] (μm)</td>
<td>D [4,3] (μm)</td>
</tr>
<tr>
<td>30</td>
<td>1.66 ± 0.99 (0.13-6.53)</td>
<td>2.63 ± 0.12 (6.06-1571)</td>
</tr>
<tr>
<td>40</td>
<td>0.34 ± 0.02 (0.10-3.85)</td>
<td>1.29 ± 0.08 (0.13-5.90)</td>
</tr>
<tr>
<td>50</td>
<td>0.81 ± 0.05 (0.17-3.19)</td>
<td>1.34 ± 0.06 (0.12-4.81)</td>
</tr>
<tr>
<td>60</td>
<td>0.85 ± 0.09 (0.21-2.73)</td>
<td>1.22 ± 0.05 (0.11-4.52)</td>
</tr>
</tbody>
</table>

Table.2.5: Particle size of powders sprayed at 5 mg/mL and 50 mg/mL concentration

<table>
<thead>
<tr>
<th>Particle size (μm)</th>
<th>Commercial Vancomycin Hydrochloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (0.9) μm</td>
<td>1413±42.72</td>
</tr>
<tr>
<td>D (0.5) μm</td>
<td>35.23±2.10</td>
</tr>
<tr>
<td>D (0.1) μm</td>
<td>2.94±0.13</td>
</tr>
<tr>
<td>D [4,3] μm</td>
<td>375.77±40.31</td>
</tr>
</tbody>
</table>

Table.2.6: Particle size of commercial vancomycin hydrochloride powder
2.3.2.4. Scanning Electron Microscopy (SEM)

The morphology of the SD and commercial powders was determined using SEM analysis. Figures 2.8 and 2.9 shows representative SEM images of commercial and SD vancomycin hydrochloride powders. The commercial powder was composed of irregularly shaped particles which formed large angular particles and cohesive aggregates that demonstrated a very broad particle size range with particles up to 1mm in size. It is seen that mechanical interlocking due to surface roughness is one of main forces in particle-particle interaction resulting in formation of aggregates which results in poor aerosol generation due to inefficient particle dispersion (14). SD powders demonstrated dramatically altered particle morphologies with spherical and porous particles. The particles produced from the spray drying process showed the presence of pores which was consistent with rapid particle drying and cavity formation. The images suggest primary particles and secondary particle aggregates were in the size range of 5 µm or
less. There were a high proportion of fine sized particles loosely agglomerated in case of powders sprayed at low concentration as compared to powders sprayed at high concentration which had a low proportion of the loose agglomerates. Thus the spray drying process yields powders which are spherical in shape, have lower area of contact and narrow particle size distribution in the respirable range which results in higher respirable fraction as compared to mechanically milled powders (8).

Figure.2.8: SEM image of commercial vancomycin hydrochloride powder 50X magnification
Figure 2.9: a) SEM image VH SD powder (5 mg/mL) at 150°C 50 mm b) VH SD powder (50 mg/mL) at 150°C 50 mm 12000X magnification

2.3.2.5. Testing the in vitro deposition efficiency of dry powder inhalation of vancomycin hydrochloride

All aerosolized vancomycin powders were recovered in the NGI according to USP guidelines and had mass balance within acceptable limits. Table 2.7 summarizes the aerodynamic particle size distribution of % FPF ≤5μm, MMAD, and GSD for aerosolized commercial and SD vancomycin hydrochloride powders. It is seen that the commercial powder has very low in vitro deposition efficiency as compared to the spray dried powders which is evident from the % FPF ≤5μm and the MMAD values. Thi et al compared the in vitro deposition efficiency of micronized and spray dried terbutaline sulphate. The percent fine particle fraction of spray dried terbutaline sulphate was
significantly greater than the micronized drug (90). The D (0.5) value of commercial vancomycin hydrochloride was approximately 35 μm and the particles exhibited rough surfaces and formed cohesive aggregates. Therefore due to presence of the large particles, commercial VH had a very high MMAD of 27 μm and subsequently 90% of the drug was deposited in the induction port and the first stage of NGI. Thus only 11 % of the drug was deposited in the stages with cut off diameter of <5 μm and which mimicked the deep airways. The spray drying process has successfully improved the dispersion properties of various drugs like tobramycin, salbutamol by modifying the particle size and morphology (91)(92). The vancomycin hydrochloride SD powders were spherical in shape and measured in the respirable size range of 1-5 μm as compared to the commercial micronized powder which was irregularly shaped and measured greater than 1000 μm. The SD powders had % FPF ≤5μm of > 50 %. The MMAD of the SD powders was in the range of 1-5 μm which is suitable for pulmonary delivery. Also the SD powders formed loose agglomerates which were evident from the SEM images. When the capsule in Aerolizer DPI device was pierced and the air was drawn into the device, the inertial force generated by the rotation of capsule was sufficient to help the loosely agglomerated drug break down and escape the capsule. Thus the SD powders were better dispersed into their primary particle size during aerosolization due to which the drug was deposited in the lower stages of NGI which corresponded to the broncho-alveolar region of the airways. The powder sprayed from 50 mg/ml feed concentration at spray flow rate of 30mm exhibited a low % FPF ≤5μm of 26 % even though the MMAD of powder was found to be 5.75 μm. The D (0.1) and D (0.5) values of this spray dried powder were approximately 6 μm and 1150 μm respectively. Due to the large particle size of the powder, more than 70
% of the drug was deposited in the inhaler and the induction port of the imapctor and thus the \( \%\) FPF \( \leq 5\mu m\) was lower than other SD powders. The MMAD value is thus calculated for the particles which were in the range of 6 \( \mu m\) and which were deposited in the lower stages of NGI. The SD and the commercial powders generated polydisperse aerosols which was evident from the GSD value which was greater than 2.

<table>
<thead>
<tr>
<th>Spray flow rates (mm)</th>
<th>MMAD (( \mu m))</th>
<th>GSD</th>
<th>% FPF( \leq 5\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Concentration</td>
<td>High Concentration</td>
<td>Low Concentration</td>
</tr>
<tr>
<td>30</td>
<td>2.86</td>
<td>5.75</td>
<td>3.11</td>
</tr>
<tr>
<td>40</td>
<td>1.78</td>
<td>4.16</td>
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</tr>
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<td>1.97</td>
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<td>60</td>
<td>3.96</td>
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</tbody>
</table>

Table 2.7: Aerodynamic parameters of powders sprayed at 5 mg/mL and 50 mg/mL

2.3.3. Conclusion

The spray drying process yielded dried powders with stable and acceptable levels of residual moisture between 7-11%. The spray drying process did not change the physical state of the SD powders as evidenced by DSC and TGA results. Spray drying process produced powders which had smaller particle size when compared with commercial powders. The SEM images exhibited that the SD powders were spherical in shape with presence of pores showed the presence of loose agglomerates which are ideal for aerosolization to the lungs. The SD powders had MMAD in range of 1-5 \( \mu m\) and the %
FPF$_{≤5 \mu m}$ ranged from 26-85 %. Spray drying process produced powders which produced heterodisperse aerosols. Thus the spray drying process was successfully used to prepare vancomycin hydrochloride powder for inhalation which had high \textit{in vitro} deposition efficiency.

2.4. Comparison of the \textit{in vitro} deposition efficiency of nebulized solution and dry powder inhalation of vancomycin hydrochloride

The aerodynamic parameters of the nebulized solution and dry powder inhalation of vancomycin hydrochloride (VH) are enlisted in Table 2.8. The powders spray dried at 150°C and 50 mm spray flow rate from feed concentration of 5 mg/mL and 50 mg/mL are used as representatives of the spray dried powders since these powders exhibited the best values for the aerodynamic parameters. It is evident from the table that the nebulized solution has a high MMAD of approximately 9 μm as compared to the spray dried powders which have MMAD in range of 2-3 μm. From the MMAD values it can be comprehended that the nebulized solution produced large sized aerosol droplets which were mainly deposited in the initial three stages of the NGI. The nebulized solution was not efficient in producing aerosols which could be deposited in the later stages of NGI which had a cut off diameter of $≤ 5 \mu m$. On the contrary, the spray dried powders produced aerosolized particles in the respirable size range of 1-5 μm, which were able to deposit in the stages of NGI which had cut off diameter $≤ 5 \mu m$. The % FPF$_{≤5 \mu m}$ of the nebulized solution and the spray dried powders correlate with the MMAD values of these formulations. The nebulized solution due to a high MMAD value, had % FPF$_{≤5 \mu m}$ of just 34 %, while the spray dried powders due to their optimum MMAD value had % FPF$_{≤5 \mu m}$ of greater than 70 %. The GSD of the nebulized and spray dried powders was greater
than 2.0 which suggested that these formulation produced heterodisperse aerosols. The in vitro deposition efficiency of the spray dried powders was also compared with the commercial powder. The commercial powder had large irregular sized particles which was evident from the particle size analysis data and SEM images. The MMAD of commercial powder was approximately 27 μm and thus subsequently had a low % FPF_{≤5 μm} value of 11.62 %. The spray dried powders had particles in the range of 0.1-5 μm and the particles were spherical in shape and showed the presence of pores. Thus the spray dried powders had MMAD in range of 2-3 μm and corresponding % FPF_{≤5 μm} greater than 70%. Comparing the MMAD of the commercial powder to the spray dried powder, it is evident that spray drying process is capable of producing powders of desired size and shape and thus produces powders with high % FPF_{≤5 μm}. The MMAD and D [4, 3] values of the powders were also compared. The MMAD determined the degree of powder deaggregation during aerosolization to generate primary particles which are deposited in the later stages of NGI. The D [4, 3] is the volume mean diameter which calculates the diameter based on volume of the particle and comparing it to a volume of sphere. The volume can be measured of a single large particle or small particles clustered together to from a large particle. Thus D [4, 3] can be used to determine the aggregation of particles. In case of powders spray dried from feed concentration of 5 mg/mL, the MMAD and D [4, 3] were closely related indicating that the aerosolization was capable of deaggregating the loosely aggregated particles into primary particles. In case of powders sprayed from feed concentration of 50 mg/mL, it can be seen that the MMAD is higher than the D [4, 3] values. This may be due to the fact that the aerosolization of this powder did not generate sufficient energy to disperse the loose agglomerates into primary particles. The
D [4, 3] values are determined by dispersing the particles in a liquid medium whereas the MMAD is determined by dispersing the powder in air and the difference in the values could be attributed to the use of different mediums.

Thus it can be concluded that the spray dried powders exhibited better *in vitro* deposition efficiency than nebulized solution of vancomycin hydrochloride. The spray drying process was efficient in producing powders of desired size and shape suitable for aerosolization. The spray dried powders had better *in vitro* deposition efficiency than commercial vancomycin hydrochloride powder.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>D [4,3] (μm)</th>
<th>MMAD (μm)</th>
<th>GSD</th>
<th>% FPF ≤5 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulized solution</td>
<td>-</td>
<td>8.89</td>
<td>2.29</td>
<td>34.01</td>
</tr>
<tr>
<td>VH SD</td>
<td>1.34</td>
<td>1.97</td>
<td>2.89</td>
<td>80.39</td>
</tr>
<tr>
<td>5 mg/mL</td>
<td>1.34</td>
<td>1.97</td>
<td>2.89</td>
<td>80.39</td>
</tr>
<tr>
<td>50 mm</td>
<td>1.34</td>
<td>1.97</td>
<td>2.89</td>
<td>80.39</td>
</tr>
<tr>
<td>VH SD</td>
<td>1.95</td>
<td>3.09</td>
<td>3.31</td>
<td>72.30</td>
</tr>
<tr>
<td>50 mg/mL</td>
<td>1.95</td>
<td>3.09</td>
<td>3.31</td>
<td>72.30</td>
</tr>
<tr>
<td>50 mm</td>
<td>1.95</td>
<td>3.09</td>
<td>3.31</td>
<td>72.30</td>
</tr>
<tr>
<td>Commercial VH</td>
<td>375.77</td>
<td>27.07</td>
<td>2.23</td>
<td>11.62</td>
</tr>
</tbody>
</table>

Table 2.8: Comparison of aerodynamic parameters of nebulized solution, commercial and spray dried powders of vancomycin hydrochloride
2.5. Determination of *in vitro* anti-bacterial activity of spray dried powders

2.5.1. Introduction

*Staphylococcus aureus* is a gram positive coccus and is a part of normal flora on the skin and the mucosal surfaces, with the squamous epithelium of anterior nares being its primary habitat (44). Normal strains of *S. aureus* are very susceptible to antibiotic treatment. However, resistant forms of *S. aureus* are especially problematic and have been associated with altered genetic make-up, specifically SCCmec. SCC stands for staphylococcal chromosomal cassette and *mec* is the gene encoding methicillin resistance. The *mec* encodes PBP2A, a penicillin binding protein which has intrinsic reduced affinity for methicillin and all β-lactams. The *mec* gene is present in *S. aureus* for it to show resistance to penicillin and cephalosporin. There are five types of SCCmec from I to V. MRSA strains found in hospital settings carry SCCmec type I-III, while SCCmec type IV is mainly found in community associated MRSA strains (44)(46). These Methicillin Resistant *S. aureus* (MRSA) strains are particularly problematic in current antimicrobial treatment regiments.

International surveillance data estimates that almost 20% of patients suffering from nosocomial pneumonia are infected with *S. aureus* with 40-60% of the *S. aureus* isolates being methicillin resistant (63). Vancomycin hydrochloride is a glycopeptide antibiotic and is currently used as the first line of therapy in treatment of Methicillin resistant *Staphylococcus aureus* infections. Vancomycin inhibits bacterial cell wall synthesis and results in slow bactericidal activity with time dependent killing (46).
2.5.2. Materials

MRSA 1371 culture was provided by Dr. Richard Goering. Dehydrated powder BBL™ Mueller Hinton Broth for preparing Muller Hinton broth was obtained from BD Diagnostic systems (Franklin Lake, NJ). Sterile Muller Hinton agar plates (60mm in diameter) were obtained from Teknova (Hollister, CA) and blood agar plates were obtained from Remel (Lenexa, Kansas).

2.5.3. Methods:

Bacterial culturing was performed according to standard procedures. Briefly, MRSA 1371 bacteria were streaked onto blood agar plates in order to isolate the MRSA colonies and incubated overnight at 37°C. Muller-Hinton broth was prepared in side arm flask and was sterilized by autoclaving the solution at 116-120°C for 10 minutes. Isolated MRSA colonies were suspended in Mueller Hinton broth by using sterile cotton swab and shaken in an incubator at 37°C till the absorbance measured by spectrophotometer (Bichrom Libra S6) at 540nm (A540nm) was around 0.4. This absorbance measurement corresponds with a bacterial stock suspension with an inoculum density of 5x10⁸ cfu/mL (cfu: colony forming units). The bacterial stock solution was then serially diluted 10 times with sterilized normal saline (0.9% NaCl) to yield an inoculum density of 5x10⁵ cfu/mL. Inoculums of 10 μL were pipetted onto the agar plates and spread uniformly using sterilized and fused glass capillary.

The stock solution of commercial vancomycin hydrochloride was prepared by dissolving 12mg in 10 mL of normal saline(120 μg/mL). The resulting drug solution was filter sterilized using 0.22 μm membrane filter and used to prepare serial dilutions 15-120
μg/mL. Drug solutions were also prepared using SD vancomycin powder processed at 150ºC and 60mm spray flow rate from 5 mg/mL and 50 mg/mL feed concentrations. Antibiotic aliquots of 100 μL were then pipetted onto the bacterial inoculated plates and uniformly spread with sterilized glass capillary. Normal saline was used as a negative control (blank). The bacterial inoculated plates were incubated at 37ºC for 24 hours. The colonies were counted visually using colony counter (New Brunswick Scientific Co, New Brunswick, NJ).

2.5.4. Results and Discussion

The bacterial colony counts for the blank and drug treated bacterial plates is presented in Table 2.9. The blank plate showed the maximum number of MRSA colonies and was used as a negative control for other plates. Antibiotic treated plates demonstrated apparent reductions in the number of MRSA colonies. Vancomycin at a 60 μg/mL concentration produced a 3 log reduction in the number of MRSA colonies. It was also seen that solutions prepared using both commercial and spray dried vancomycin at 120 μg/mL showed complete inhibition in growth of MRSA colonies. Therefore, the SD process did not appear to impair the antibacterial activity of vancomycin.

The minimum inhibitory concentration (MIC) range of vancomycin for MRSA is reported to be 0.5-2.0 μg/mL (58). However, MIC values are determined in broth and do not directly correlate to the concentration of vancomycin in a 100μL plated drug volume. Therefore, the amount of drug rather than the concentration of vancomycin was evaluated to completely inhibit the MRSA colonies (Table 2.9). Based on NGI studies, the minimum amount of vancomycin hydrochloride deposited on a single stage was 90 μg
Therefore, the minimum amount of vancomycin hydrochloride deposited in the NGI was greater than the amount of vancomycin required for complete inhibition of MRSA growth.

The elevated temperature and pressures associated with spray drying of a peptide antibiotic were demonstrated to be insignificant to impair the antimicrobial activity of spray dried vancomycin. Generally, the spray drying outlet temperature is the maximum temperature that a product can reach with reported processing temperatures of up to $80^\circ$C being associated with retained antimicrobial activity (93).

2.5.5. Conclusion

It was seen that 12 μg of commercial vancomycin hydrochloride showed complete inhibition of MRSA colonies. The drug powders sprayed at extreme spray drying conditions also exhibited complete inhibition of bacterial colonies at the mass of 12 μg. Thus the spray drying temperature did no degrade vancomycin hydrochloride and the spray dried powders retained their anti-bacterial activity.
<table>
<thead>
<tr>
<th>Treatment (μg/mL)</th>
<th>Treatment (μg)</th>
<th>Number of MRSA colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td></td>
<td>409</td>
</tr>
<tr>
<td>15</td>
<td>1.5</td>
<td>227</td>
</tr>
<tr>
<td>30</td>
<td>3.0</td>
<td>68</td>
</tr>
<tr>
<td>60</td>
<td>6.0</td>
<td>2</td>
</tr>
<tr>
<td>120</td>
<td>12.0</td>
<td>0</td>
</tr>
</tbody>
</table>

0*: Control (Normal Saline)

Table 2.9: Therapeutic efficacy of vancomycin hydrochloride solution on MRSA colonies
Chapter 3

Specific Aim ‘B’:

Developing a proof of concept for an *in vitro* model using 
Next Generation Impactor as a potential screening tool 
for aerosolized antibiotics
3.1. Introduction

The pulmonary absorption, permeability, disposition, efficacy and toxicity of inhaled formulations can be assessed by using different in vitro methods (94). In vitro methods offer simplicity, robustness and better control while performing experiments and during data acquisition as compared to in vivo methods. In vitro methods also have benefits of being reproducible and have better dosing control. These studies also help in reducing operational costs and minimizing or eliminating costs associated with animal experimentation. New formulation optimization for pulmonary delivery is limited in part due to inadequate in vitro models which can be used to mimic conditions in the lungs. Immortalized cell lines such as like Calu-3, 16HBe14o-, CFBE41o-, and A549 as well as primary human airway cell culture like small airway epithelial cells (SAEC’s) and the human alveolar epithelial cells (hAEpC) are representative of cell-based models to predict in vivo absorption across airway epithelial cells (95, 96). In the case of lung delivery, the drug is deposited on the cell layer as aerosolized solid particle or liquid droplets. However, in vitro methods typically apply drug solutions or suspensions directly to the cell layers. The process of in vivo particle dissolution in the lungs varies significantly when the drug solution is spread on to the in vitro cell layers and when the drug solution is aerosolized onto the in vitro cell layers. The drugs which are applied as solution form a uniform concentration across the cell layer. Deposition of aerosolized drug particles or droplets on to the cell layer establishes a non-uniform concentration gradient compared to the uniform gradient established by the simple addition of drug solutions to the cell layer (97). As previously discussed, cascade impactors are used to determine the particle size distribution of aerosols and can be related to a simple model of
the lung. Some reported studies have examined aerosol absorption, dissolution and toxicity by placing the \textit{in vitro} cell models inside specific stages of cascade impactor and impinger. Fiegel et al. aerosolized large porous particles onto the surface of Calu-3 cells grown on transwell filters using a liquid impinger. The study used different culture conditions such as air-interface culture (AIC) and liquid covering culture (LCC) to determine the effect of culture conditions on the expression of cellular junction proteins and on the integrity of Calu-3 cells impinged with aerosols. It was concluded that AIC grown Calu-3 cells served as an appropriate \textit{in vitro} model for characterization of therapeutic aerosols (98). Cooney et al. used Calu-3 and SAEC’s cell layers which were grown on transwell inserts and placed in the Andersen 8-stage viable cascade impactor. Fluorescein isothiocyanate (FITC) – dextran solutions were used as marker compounds and nebulized onto the cell layers. It was seen that the permeability of FITC-dextran across Calu-3 and SAEC cells mimicked the \textit{in vivo} clearance of these molecules from animal lungs (96). The two studies mentioned above did not take into consideration the turbulence or altered airflow through the device caused by the presence of transwell filters during aerosol deposition. Also in these studies, the cascade impactors were not modified to incorporate the inserts on which cells were grown. Bur et al. tried to minimize the turbulence caused by the air stream by integrating upside down transwell filters on which Calu-3 cell were grown in the multi-stage liquid impinger (MSLI). The second and third stages of the MSLI were modified to accommodate the cell-containing inserts. They reported no significant change in the deposition patterns of nebulized fluorescin-sodium solution in the modified MSLI and the non-modified MSLI (99). Despite these previous reports of incorporating cell cultures in aerosol testing devices,
these studies focused on the process of pulmonary drug absorption. However, no reports have evaluated cell-based pharmacodynamic effects while simultaneously evaluating aerosolization parameters and processes. Specifically, no reported *in vitro* model has incorporated bacterial cultures into cascade impactors to test the antibacterial efficacy of aerosolized antibiotics. The main aim of this study was to develop a proof of concept of an *in vitro* model of NGI which can be used as potential screening tool for aerosolized antibiotic formulations. The principal objective of this study was to modify deep collection cups to incorporate bacterial cultures so that they could be used in place of the normal collection cups while minimizing airflow disturbances. Modified deep collection cups were used to test the *in vitro* deposition efficiency and the *in vitro* anti-bacterial efficacy of the nebulized vancomycin hydrochloride solution.

3.2. Testing and comparing the *in vitro* deposition efficiency of nebulized solution using normal and modified deep collection cups of NGI

3.2.1. Materials and methods

The normal collection cups were obtained along with the NGI from MSP Corporation (Shoreview, MN). The depth of the normal collection cup is specified in the USP. The deep collection cups were custom made for the purpose of this experiment. Solid aluminum blocks were milled to the dimensions of the normal collection cups but with sufficient cup depth to accommodate cell cultures and petri plates. The purpose for using the deep collection cup was to incorporate agar culture plates in the cups without affecting the dimensions of the collection cups as specified by USP. Sterile Muller Hinton agar culture plates (60mm in diameter) were obtained from Teknova (Hollister, CA). The distance from the cup lip to the top of the agar surface within the modified deep
collection cup was adjusted using thin plastic sheets to the value specified in the USP. The depth of the deep cup, the thickness of the agar filling in the culture plate, and the thickness of the plastic sheets were measured by using electronic digital calipers. Since a proof of concept to develop an *in vitro* model using the NGI was being established, the normal collection cup in only the fourth stage of NGI was replaced with the modified deep collection cup. Vancomycin hydrochloride solution (VH solution) was prepared by dissolving 40mg of vancomycin hydrochloride in 10mL of normal saline and 5mL of VH solution was used for nebulization. The nebulization of drug solution was carried out using the NGI which was operated at 15 L/min as described in section 2.3. Two sets of experiments were carried out; in the first set the VH solution was nebulized using normal collection cups in all the stages of NGI, while in the second set the normal collection cup in the fourth stage was replaced with modified deep cup and all other stages had normal collection cups and VH solution was nebulized. The first set of experiments was carried in triplicates while the second set of the experiment was carried in duplicates. In the modified deep collection cup, an aluminum foil sheet with negligible thickness was placed on the top of agar layer of the culture plate. This was done mainly to ensure complete recovery of the drug from the agar plate and prevent diffusion of the drug in the agar layer. Drug recovery was performed and analyzed according to methods described previously. The percentage of total drug recovered is determined as follows:

\[
\% \text{ Dose Recovered} = \frac{\text{Amount of drug recovered from nebulizer and NGI parts}}{\text{Amount of drug aerosolized}} \times 100
\]

The total amount of drug dose deposited in the induction port and the NGI collection cups was determined and was used to calculate the percentage of drug dose deposited in
each stage of NGI. The percentage of dose deposited in each stage for the two sets of experiments was statistically compared by using the student t-test: two samples assuming equal variance. The *in vitro* deposition efficiency was determined from the percentage of dose deposited in each stage of the NGI. While recovering the drug deposited in modified deep collection cup placed in stage 4 of NGI, the aluminum sheet on which the drug droplets were deposited was washed with aqueous mobile phase. The agar plate and the deep collection cup were also separately washed to determine if there was any drug deposited on them.

3.2.2. Results

The normal collection cup has a depth of 14.625 ± 0.10 mm as specified in the USP. The deep collection cup had a depth of 24.43 ± 0.08 mm. While the thickness of the agar layer in the petri plate and plastic sheets was found to be 9.24 mm. The representative pictures of normal and modified deep collection cup is depicted in Figure 3.1.

Figure 3.1: a) Normal collection cup b) Modified deep collection cup
The deep collection cup was placed in only the fourth stage of NGI because when the NGI is operated at flow rate of 15 L/min the cut-off diameter is ≤5 μm from stage 4 to stage 7. The percentage of total vancomycin hydrochloride recovered from the NGI and the nebulizer after nebulization using the normal collection cups was found to be 93.88 ± 3.61 %. The percentage of total vancomycin hydrochloride recovered from the NGI and the nebulizer after nebulization using the deep collection cup was found to be 92.50 ± 2.08 %. The percentage of dose deposited in only NGI stages using normal collection cups and modified deep collection cup is given in Figure 3.2.

3.2.3. Discussion

Equivalent dose recoveries were observed for both normal and deep collection cups. On the fourth stage of NGI where the normal collection cup was replaced by modified deep cup, no drug was found to be deposited in the deep cup and the agar plate with the entire drug deposited onto the aluminum foil sheet. Drug did diffuse into the agar layer and was recovered completely from the stage. The culture plates were of appropriate diameter such that all the drug droplets existing from the nozzle got deposited into the culture plate. The manufacturers of the cascade impaction device provide a definitive calibration for the separation characteristics of each impaction stage in terms of the relationship between the stage collection efficiency and the aerodynamic diameter of particles and droplets passing through it as an aerosol. Device calibration is dependent on the orifice dimensions, spatial arrangement of the nozzle and its collection surface, and airflow rate passing through it. Stage mensuration is performed which ensures that the impactor conforms to the specified critical dimensions set for the instrument for effective testing of inhaler products. The critical dimensions for the NGI are provided in the USP (9).
3.3 shows that cup depth (dimension b) and the stage nozzle to seal body distances for each stage (dimension c) are the critical dimensions to be considered when replacing the normal collection cups with modified deep collection cup. The main challenge in modifying the deep cup was to maintain the critical dimension b and c as compared to the normal collection cup. The cross section of normal and modified deep collection cups is shown in Figure 3.3. In case of the modified deep collection cup, the dimension b was altered to accommodate the petri plates but was adjusted using thin sheets of plastic in order to maintain the dimension b for accurate and effective testing of the aerosol. The percentage of dose deposited in only the NGI stages were compared for the experiment carried with normal cups and the experiment carried with modified deep cup. It was seen that there is no statistically significant difference (p>0.05) in the percentage dose deposited in modified deep collection cup in stage 4 of NGI and the percentage dose deposited in normal collection cup in stage 4 of NGI. The \textit{in vitro} deposition efficiency of the nebulized solution was significantly similar in case of normal and modified deep collection cup. This proved the fact that, plastic sheets and agar culture plate were successful in adjusting the depth of the deep collection cup and the modified deep collection cup had similar depth as that of the normal collection plate. It was also seen there was no statistically significant difference (p>0.05) in the percentage of dose deposited in the induction port and other stages of NGI where only normal collection cups were used. However there was a statistically significant difference (p<0.05) in the percentage of dose deposited in stages 2 and 5 of NGI where only normal collection cups were used. This difference could be due to the fact that only fourth stage was replaced with the modified deep cup and for other stages normal cups were used. The statistical
difference may be also due to the limited number of sampling points used for comparison of the percentage of dose deposited in the NGI stages. Further studies need to be carried out where modified deep collection cups are used in all the stages of the NGI. The dose deposited in modified cup for each stage of the NGI should then be statistically compared with dose deposited in normal cup for each stage of NGI.

Figure 3.2: Percentage of dose deposited in NGI stages only using the normal and modified deep collection cup

*Indicates statistical significance (p<0.05)
Figure 3.3: Cross section of a) Normal collection cup b) Modified deep collection cup

3.2.4. Conclusion:

The deep collection cup was successfully modified by using plastic sheets and agar plate. There was no significant difference in the percentage dose deposited in modified deep collection cup and the percentage dose deposited in normal collection cup. The \textit{in vitro} deposition efficiency in normal and modified collection cup was statistically similar.

3.3. Testing the \textit{in vitro} anti-bacterial efficacy of nebulized solution using modified deep collection cup of NGI

3.3.1. Materials and methods

The plastic sheets and agar culture plate were used to modify the deep collection cup as discussed in section 3.2. The modified deep collection cup was placed in only the fourth stage of NGI in place of the normal cup. Normal collection cups were placed in all other stages of NGI. The agar plates were inoculated with MRSA culture in a similar manner as discussed in section 2.6. The bacterial culture plates were divided into three types: a positive control plate, a negative control plate and test plate. The bacterial inoculated
plates were treated by nebulizing the drug solution and spreading the drug solution on to the plates. The amount of drug deposited in the modified deep collection cup placed in stage 4 of NGI was found to be 1.39 mg. The solution of vancomycin hydrochloride was prepared by dissolving 69.5 mg of the powder in 5 mL of normal saline (13.9 μg/mL). The resulting solution was filter sterilized using 0.22 μm membrane filter. 100 μL of this solution was then pipetted onto the bacterial inoculated plate and uniformly spread with sterilized glass capillary and this was the positive control plate. 100 μL of normal saline was pipetted onto the bacterial inoculated plate and uniformly spread with sterilized glass capillary and this was used as a negative control plate which served as the blank control. Vancomycin hydrochloride solution (VH solution) was prepared by dissolving 40mg of vancomycin hydrochloride in 10mL of normal saline and 5mL of VH solution was used for nebulization. The bacterial inoculated agar plate which was the test plate was placed inside the modified stage 4 of the NGI. The nebulization of drug solution was carried out using the NGI which was operated at 15 L/min as described in section 2.3 and this experiment was carried in duplicates. The bacterial inoculated plates were incubated at 37°C for 24 hours. The colonies were counted visually using colony counter.

3.3.2. Results and Discussion

The number of colonies in negative control plate was found to be 236 ± 11. There were no colonies found in the positive control plates and test plates treated with nebulized drug solution. The fourth stage of NGI was used as a representative stage for developing the proof of concept for an in vitro NGI model. The fourth stage was selected for modification because at flow rate of 15 L/min the cut-off diameter for stage 4 was ≤ 5 μm. In order to deliver the aerosol in the respiratory bronchioles and the alveoli, the
optimal respirable size range is 1-5 μm. It was seen that the nebulized vancomycin hydrochloride solution deposited an adequate amount of drug on bacterial test plate to inhibit the growth of MRSA colonies. An equivalent amount of drug deposited in the modified collection cup was spread manually on the bacterial inoculated plate which was used as the negative control. It was observed that the nebulized drug solution had similar effect in inhibiting the growth the MRSA colonies as that of drug solution spread on the bacterial inoculated plates. The *in vitro* anti-bacterial efficacy of nebulized vancomycin hydrochloride was demonstrated by placing the bacterial inoculated plates placed inside the NGI. This provided proof to the concept of developing an *in vitro* model for testing the anti-bacterial efficacy of aerosolized antibiotics.

3.3.3. Conclusion

The bacterial culture plate placed in the modified collection cup in stage 4 of NGI showed complete inhibition of MRSA colonies when compared to the negative control plate. The effect of the nebulized antibiotic on bacterial colonies was similar to the effect produced by spreading the antibiotic solution onto the bacterial plates; there was complete inhibition of MRSA colony growth. A proof of concept was successfully developed to test *in vitro* anti-bacterial efficacy of nebulized solution using modified deep collection cup of NGI.
Chapter 4

Summary and Future directions
4.1. Summary

A rapid analytical method for vancomycin hydrochloride quantification was developed and validated using Ultra Pressure Liquid Chromatography and standard validation protocols. The aqueous mobile phase (A) consisted of 95:5 (v/v) 5mM KH2PO4 in water and acetonitrile and the apparent pH adjusted to 3.0 with phosphoric acid. The organic mobile phase (B) consisted of 100% (v/v) acetonitrile. Specifically, vancomycin hydrochloride was eluted using Acquity UPLC BEH C18 1.7 μm 2.1 x 100 mm column at flow rate of 0.4 mL/min under gradient conditions. This UPLC method was used to quantify the amount of vancomycin deposited on stages of the Next Generation Impactor (NGI). The osmolality and pH of vancomycin hydrochloride solution were found to be 291.67 ± 0.58 mOsm/kg and 4.26 ± 0.01 respectively and were within the acceptable ranges for administration of aerosolized solutions to the lungs. The in vitro deposition efficiency of nebulized drug solutions had a high MMAD value of 8.89 μm and low % FPF≤5 μm of 34.01 %. Spray dried vancomycin powders yielded dry powders with no change in the physical state of vancomycin hydrochloride after processing. The spray drying process produced powders which had narrower particle size distributions when compared with commercial powder. The SEM images showed porous spherical particles that seemed to form loose agglomerates. The spray dried powders had MMAD in respirable range of 1-5 μm and the % FPF≤5 μm ranged from 26-85 %. The in vitro deposition efficiency of nebulized solutions and dry powder inhalation of vancomycin hydrochloride was compared. The in vitro deposition efficiency of dry powder inhalation was better than the nebulized solution. Both nebulized solutions and aerosolized powders had polydisperse particle size distribution and GSD values> 2. The processing conditions
did not affect the anti-bacterial activity of spray dried vancomycin. The *in vitro* deposition efficiencies at a single NGI stage were compared using a normal and modified deep collection cup. There was no statistically significant difference (p>0.05) observed in the percentage of dose deposited in normal and modified collection cup. The modified collection cup was successfully used to test the anti-bacterial efficacy of the nebulized solution. A proof of concept for an *in vitro* NGI model which can be used for testing the *in vitro* deposition efficiency and the anti-bacterial efficacy of aerosolized antibiotics was successfully developed.

In conclusion high potency spray dried vancomycin hydrochloride powders were prepared which had lower MMAD and better % FPF than nebulized solution of vancomycin hydrochloride. A proof of concept was successfully developed for an *in vitro* NGI model which could be used as a potential screening tool for aerosolized antibiotics. This research has helped to provide *in vitro* data which can be utilized for selecting optimized formulations which can be tested in *in vivo* pneumonia models. The *in vitro* NGI model after complete optimization can be used as screening tool for new inhalable antibiotic formulation before the antibiotics can be tested *in vivo*. This model will act as two-in-one equipment which can test the *in vitro* deposition efficiency and the antibacterial efficacy of the antibiotics.

4.2. Future directions

The long term experiments should focus on testing the permeability and toxicity of optimized spray dried powders of vancomycin hydrochloride using pulmonary cell cultures like Calu-3, A549. The optimized spray dried powders could also be tested for
the *in vivo* deposition efficiency and anti-bacterial efficacy using mouse and rabbit models for pneumonia. Histopathological analysis of excised animal lungs should be performed before and after the administration of the aerosolized antibiotic.

The future studies could also focus on optimization of the *in vitro* NGI model where the normal collection cups in all the stages are replaced by the modified deep collection cups. The percentage of dose deposited in the normal and modified cups should be statistically compared. The NGI model should be optimized using dry powder inhalation and nebulized solution of different antibiotics. The *in vitro* model should also be optimized using gram negative bacteria such as *Pseudomonas aeruginosa*, fungi and viruses. The bacterial resistance mechanisms can be studied using the optimized NGI model. Lung cell lines like Calu-3, A549 and *ex vivo* isolated lung models can also be incorporated in the *in vitro* NGI model for studying the permeability of aerosolized drugs.
References:


26. Training Papers Spray Drying, Buchi Labortechnik AG, Switzerland.


