UNIVERSITY OF PERUGIA
FACULTY OF PHARMACEUTICAL SCIENCES
PHARMACEUTICAL TECHNOLOGY

ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
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Στυλιανή Ξηρουδάκη

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ΠΕΡΙΛΗΨΗ

Η ποιότητα των κόνεων προς εισπνοή περιορίζεται από τις κακές ρεολογικές ιδιότητες των δραστικών συστατικών. Στην πλειοψηφία των περιπτώσεων, απαιτείται η ενσωμάτωση στη συνταγή κατάλληλων φορέων οι οποίοι βελτιώνουν τις ρεολογικές ιδιότητες των κόνεων. Ωστόσο, οδηγούν σε διάλυση του φαρμάκου κι έτσι αυξάνεται η ολική ποσότητα κόνεως προς εισπνοή, γεγονός που επηρεάζει θεραπείες στις οποίες απαιτούνται υψηλές δόσεις δραστικής ουσίας, όπως είναι η αντιβιοτική θεραπεία. Επιπρόσθετα, το περιορισμένο φάσμα των εγκεκριμένων εκδόχων για πνευμονική χορήγηση δυσχεραίνει την ανάπτυξη νέων εισπνεόμενων φαρμακευτικών προϊόντων. Επομένως, προτείνουμε την ανάπτυξη μιγμάτων συστημάτων χορήγησης δραστικών ουσιών με σκοπό την αποφυγή της διάλυσης των δραστικών συστατικών και ταυτόχρονα τη βελτίωση των αεροδυναμικών ιδιοτήτων των ξηρών κόνεων προς εισπνοή.

Πραγματοποιήθηκε η παραγωγή μικροσωματιδίων λευκίνης και βιοαποικοδομήσιμου πολυμερούς για την άμεση και παρατεταμένη αποδέσμευση των δραστικών συστατικών αντίστοιχα. Η παραγωγή των μικροσωματιδίων έγινε με τη διεργασία της ξήρανσης με ψεκασμό με ταυτόχρονη ενκαψυλίωση ενός αντιβιοτικού συμπλόκου και ενός αντιφλεγμονώδους φαρμάκου. Στη συνέχεια, πραγματοποιήθηκε η ανάμειξη των δύο συστημάτων, ενώ η εφαρμογή πειραματικού σχεδιασμού συνέβαλε στην κατανόηση της επίδρασης των παραμέτρων της διεργασίας στα χαρακτηριστικά ποιότητας των παραγόμενων σωματιδίων. Υπολογίστηκαν η εκπεμπόμενη δόση από τη συσκευή (Emitted Dose), το κλάσμα της εκπεμπόμενης δόσης των σωματιδίων με αεροδυναμική διάμετρο μικρότερη από 5μm (Fine Particle Fraction), τα ρεολογικά χαρακτηριστικά καθώς και η ομοιομορφία περιεχομένου των δραστικών συστατικών στα μίγματα.

Επιβεβαιώθηκε η επίδραση της λευκίνης και του πολυμερούς στην αποδέσμευση των δραστικών συστατικών. Η ενσωμάτωση της λευκίνης στη συνταγή οδήγησε σε σημαντική βελτίωση των αεροδυναμικών ιδιοτήτων των μιγμάτων. Η εκπεμπόμενη δόση από τη συσκευή ήταν σε όλα τα μίγματα υψηλή (>95%).

Συμπερασματικά, οι ικανοποιητικές αεροδυναμικές ιδιότητες και το διπλό μοτίβο αποδέσμευσης των δραστικών συστατικών της προτεινόμενης συνταγής την καθιστούν μια ενδιαφέρουσα πρόταση στα πλαίσια της πνευμονικής χορήγησης αντιβιοτικών φαρμάκων.

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NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS
FACULTY OF PHARMACY
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MULTIPLE DRUG DELIVERY MIXTURES AS A POTENTIAL STRATEGY TO IMPROVE THE AERODYNAMIC PERFORMANCE OF DRY POWDERS FOR INHALATION

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ABSTRACT

Quality of inhalation powders is restricted by the poor solid-state properties of active ingredients. This issue requires the use of proper carriers which, although they improve rheological characteristics, have a dilution effect on the drug thus demanding higher amounts of powder to be inhaled. This affects significantly therapies limited by high dosages, such as antibiotic therapy. Furthermore, the scarcity of approved excipients for pulmonary administration poses additional problems in inhaled product development. Therefore, we propose the use of multi-drug delivery system blends as a strategy to prevent drug dilution effect and simultaneously grant the required aerodynamic properties.

Slow release and fast/immediate release microparticles were prepared using a polylactide polymer and L-leucine. These systems were combined to achieve fast systemic absorption and sufficient local retention. A capreomycin/DCA antibiotic complex and an anti-inflammatory drug were co-encapsulated using a two-fluid nozzle and piezoelectric spray-drying technologies. The obtained powders were blended using a cochlea mixer at room temperature for variable times. A 2-level factorial design was built to model the aerodynamic behavior of the blends. Content uniformity, emitted dose (Aerolizer® DPI) and fine particle fraction were assessed by HPLC and a twin-stage impinger. *In vitro* release was investigated on the best powder blend.

Leucine particles successfully improved the fine particle fraction of the blends, although the emitted dose was always > 95%. Mixing time and amount of PLA powder were both influential factors. The best blending combination resulted the 30/70 PLA/leucine, when mixed for 15 minutes, either in terms of content homogeneity (%RSD = 2.8) or fine particle fraction (>50%). Suitable process conditions for obtaining inhalable multi-drug delivery blends were determined. Suitable blends showed the expected dual release pattern and excellent aerodynamic behavior, which could be extremely advantageous especially in inhaled antibiotic therapy.

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TABLE OF CONTENTS

1.ABBREVIATIONS

API	active pharmaceutical ingredient
CFC	chlorofluorocarbon
COPD	chronic obstructive pulmonary disease
CQA	critical quality attribute
CS	capreomycin sulfate
DCA	deoxycholic acid
DPI	dry powder inhaler
ED	emitted dose
FPD	fine particle dose
FPF	fine particle fraction
GI	gastrointestinal
HIP	hydrophobic ion-pair
HPLC	high-performance liquid chromatography
HPMC	hydroxylpropylmethylcellulose
MDR-TB	multiple drug-resistant tuberculosis
MIC	minimum inhibitory concentration
MMAD	mass median aerodynamic diameter
MPs	microparticles
PDD	pulmonary drug delivery
PLA	polylactic acid
pMDI	pressurized metered-dose inhaler
QbD	quality by design
QC	quality control
RP	reverse phase
SCF	supercritical fluid
SFD	spray freeze drying
SR	sustained release
TSE	transmissible spongiform encephalopathy
VHC	valved holding chamber

2. INTRODUCTION

2.1 The anatomy of lungs

The structure of the respiratory system is uniquely adapted to its basic function, which is the transport of gases inside and outside the human body. The airflow through the respiratory system can be divided into three interconnected areas: the upper airway, the airways, and the alveolar airway (also known as pulmonary parenchyma).

The upper airways consist of the systems through which the air enters: the nasal cavity and the mouth leading to the pharynx. The upper respiratory tract is completed in the larynx which extends to the lower part of the pharynx (Barrett et al., 2012).

The airways start from the trachea where they are branched, increasing the surface of the tissue in the lungs. They can be divided into two distinct zones: the conducting airways and the respiratory airways (Giovagnoli et al., 2017a). The tracheobronchial or conducting airways-the first 16 generations- which transfer the gases to and from the upper airways are the bronchi, bronchiole and terminal bronchiole.

After the trachea, the airways bifurcate about 17 times before leading to the respiratory zone. These last 7 generations, where the exchange of the gases occurs, are structured by the respiratory bronchioles, alveolar ducts and alveolar sacs (Figure 2.2).

The airways have been likened as a tree since the trachea, bronchi and bronchioles look like the trunk and branches, whereas the respiratory zone can be compared to the leaves (Patton and Byron, 2007).

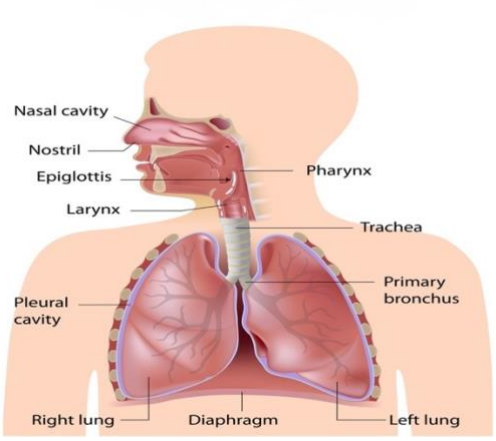


Figure 2.1 The respiratory system.

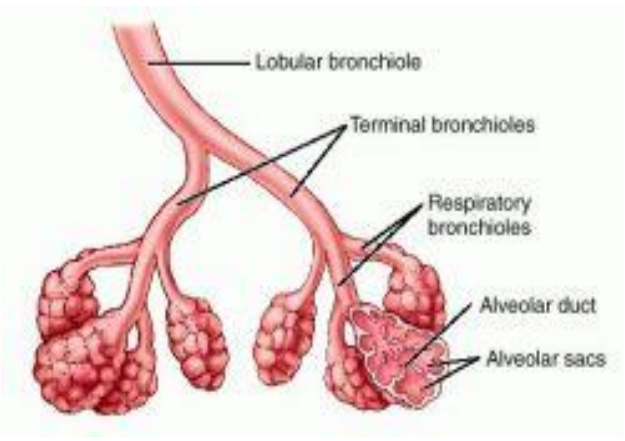


Figure 2.2 The respiratory airways.

2.2 The pulmonary drug delivery

2.2.1 Historical perspective

Ancient people had started discovering the therapeutic effects of various medicinal plants simply by observation and previous knowledge. The innovative idea of the inhaled drug therapy was first described in Ayurvedic medicine about 4.000 years ago and was based on the use of vapors or smokes. The smoke either of the leaves of the *Atropa Belladonna* plant or of a paste fixed into a pipe including *Datura species*, which contain potent alkaloids with anticholinergic bronchodilation properties, was inhaled in order to treat diseases of the throat and chest. Such pipes have been revealed to have been used also by native populations in South America around 2.000 years ago. Plenty of other ancient cultures gradually started to create the foundations for the inhaled therapy (Bisgaard et al., 2001). The oldest known method used for fumigation by the ancient people was the *incense*, a mixture of medicinal plants that was burnt and inhaled during the religious ceremonies. Later, the Greeks adopted the use of the incense whereas the Greek physician Galen described the use of powdered drugs for inhalation and Hippocrates introduced the use of vapors from herbs and resins which entered the lungs through a tube. The Romans created public baths which were later modified by the Arabs, during the eighth century, into medical centers for the treatment of various body troubles. The Arab physicians are considered to be the inventors of the general inhalation anesthesia. The great Rhazes used a sponge impregnated with narcotic plants for the anesthetic procedure before the surgical operations. The word inhaler was first introduced by the English physician John Mudge (Sanders, 2007). He described his device for inhaling opium vapor for the treatment of cough in 1778 in his book named A Radical and Expeditious Cure for a Recent Catarrhous Cough. Fourteen years earlier, in 1764, Philip Stern had highlighted the importance of the development of specific devices properly adapted to the mouth, noting: The only possible way of applying medicines directly to the lung is through the windpipe'' (de Boer et al., 2017). These resulted to the production of numerous ceramic inhalers since the 19th century. In 1863, Nelson's inhaler was described; was manufactured by S. Maw and Sons. In France, in the mid-1800s the first atomizers (also known as nebulizers) were developed. In 1858, Dr Sales-Girons, influenced by the widely-used spa therapy of that period in France, invented a portable nebulizer, named pulverisateur, which used a pump handle to draw liquid from the reservoir and force it through a nozzle against a plate. Nevertheless, thanks to chemical studies carried out by Demarquay and his colleagues, it started to become doubtful if such spray solutions

actually reach to the lungs or not. In the 1930s, the first hand-bulb nebulizers made their appearance such as the glass- bulb nebulizer supplying an adrenalin chloride solution and the plastic-bulb nebulizer. The revolution in the inhaler design was the development of the first pressurized metered-dose inhaler (pMDI), by Riker Laboratories in 1956 utilizing chlorofluorocarbon (CFC) propellants (Medihaler®,Riker). About fifteen years later, in 1970, the first dry powder inhaler/DPI (Spinhaler®,Aventis) reached the market. In 1987, the Montreal Protocol which banned the use of chlorofluorocarbon propellants, opened the road to the development and evolution of DPIs (Anderson, 2005; Frijlink and De Boer, 2004).

2.2.2 Drug delivery to the lungs- Advantages

The anatomical and physiological properties of the whole respiratory system contribute to the main advantages of this drug delivery target. The branches that characterize the respiratory zone result in a large absorptive area of up to 100 m^2 whereas the highly vascularized thin (0.1-0.2 μm) epithelial membrane can provide satisfying access of drug to blood circulation (Sunitha et al., 2011). This is the reason why the PDD is considered to be a favorable route of administration for both systemic and local actions. The localized therapy in the lungs is highly desirable for the treatment of pulmonary diseases such as pneumonia, asthma, COPD and lung cancer or even of infectious diseases which start developing in the lungs and require an antibiotic or combinational therapy e.g. bronchiectasis, tuberculosis, cystic fibrosis. In these cases, the targeted therapy not only enhances the bioavailability, due to the deposition of drugs at high concentrations in the lungs, but also reduces the possible side effects produced by other systems of drug delivery. The local targeting achieved by the pulmonary route permits the decrease of the total given dose, whereas the local drug concentration remains high, an interesting benefit for the antibiotic administration (Moghaddam et al., 2013). The optimum dose is among the most important challenges concerning the antibiotic therapy since it has to be high enough in order to reach the minimum inhibitory concentrations (MIC) for the bacteria's extermination. Hence, reducing the total dose given for the manifestation of the antimicrobial effect results in the diminution of systemic side effects for which the antibiotics are notorious (Hoppentocht et al., 2014). The increased bioavailability is a crucial benefit of the pulmonary drug delivery due to the fact that more than 40% of new drugs have low bioavailability through the oral route. This happens because in the pulmonary route, factors such as solubility, dissolution rate, permeation through the membranes and bio-stability are

less important. Consequently, it can be also used for water-insoluble drugs. Other categories of drugs that can be favored by the pulmonary route of administration could be either those that induce GI (gastrointestinal) irritation or those which may degrade in the harsh conditions of GI tract, such as large peptides and proteins (Giovagnoli et al., 2017a). In addition, drugs with poor and irreproducible bioavailability due to the effect of the first-pass metabolism in the liver as well as drugs for which a quick onset of action is required (e.g. morphine, fentanyl), can benefit from this route of administration (Frijlink and De Boer, 2004). Last but not least, the ease and convenience of administration, the fact that it is a non-invasive method combined with the lower frequency of administration can increase the patients' compliance to the therapeutic regimen.

2.2.3 The development of inhalation devices

It is widely accepted that even for potent molecules or optimized formulations, the in vivo performance of marketed inhaled products could be modified depending on the device used. A successful pulmonary administration requires a harmonic interaction between the formulation, inhaler device and patient. The effectiveness of inhaled therapy is highly dependent on the amount of aerosol that reaches the desired site of deposition and the deposition pattern of the administered aerosol is substantially influenced by the interaction and the co-operability of both the formulation and delivery device (Ibrahim et al., 2015). A characteristic example which can be considered as strong evidence of such an interaction is the first inhaled insulin Exubera ® DPI, which was withdrawn in 2007 because of poor patient compliance due to the cost and bulkiness of the device (Price et al.2018). The properties that the ideal device should have are presented below.

Each device used for inhalation therapy should be:

- ✓ Effective, such as generate an aerosol of suitable size (ideally within the range 0,5-5 μm)
- ✓ Reproducible
- ✓ Precise
- ✓ Able to retain the drug formulation chemically and physically stable
- ✓ Easy to use
- ✓ Environmentally friendly
- ✓ Affordable (Dal Negro, 2015).

The pulmonary drug delivery is mainly classified into three distinct classes:

- Nebulizers
- Pressurized metered dose inhalers (pMDIs)
- Dry powder inhalers (DPIs) (Sahane et al., 2012)

Each class presents unique strengths and weaknesses (Pilcer and Amighi, 2010).

This classification is based on the physical state of either the dispersed phase or the continuous medium. In particular, the substantial difference that distinguishes the nebulizers from pMDIs is the fact that the drug is dissolved or suspended in a polar liquid, mainly water. There are two types of nebulizers, jet and ultrasonic, which differ in the force used to convert the drug solution or suspension into fine droplets, which are then inhaled by the patient over a couple of minutes (Ibrahim et al., 2015).

Nebulizers are mainly used in hospital and ambulatory care settings due to their large size which comprises one of the main disadvantages of such delivery systems. The limited portability and the fact that the aerosol is delivered continuously over an extended period of time are the reasons why they are not typically used for the cure of chronic diseases.

The pMDIs are devices which contain solid drug dissolved or suspended in a nonpolar volatile propellant and disperse the aerosol in precise measured amounts. Compared to nebulizers, they are easier to handle, faster and less noisy (de Boer et al., 2017). Nevertheless, regardless of whether the drug is formulated as a solution or suspension, several issues arise, like low solubility and chemical stability of the active pharmaceutical ingredient (API) or instability of the suspension, respectively, leading to inaccurate dose metering or limited drug loading capacity (Berkenfeld et al., 2015). In addition, they emit the dose in high velocity resulting in important oropharynx deposition and thus their use is often limited to the treatment of upper airway conditions. Their effectiveness is highly influenced by the patients' education since they require careful co-ordination of actuation and inhalation, a function that becomes especially difficult for children and the elderly. Despite the efforts done to enhance their performance, such as the introduction of spacers and valved holding chambers (VHCs) in order to reduce the oral deposition of the inhaled particles, the incorrect use of the pMDIs still remains a prevalent issue. The fact that they include a propellant not only limits the use in drugs that are stable in certain propellants but also render them environmentally unfriendly when the chlorofluorocarbons (CFCs) are used, since they induce the depletion of ozone layer (Sahane et al., 2012).

For the past thirty years, the pulmonary drug delivery has been dominated by the pMDIs, which are believed to account for more than 80% of the global market, concerning the inhaled pharmaceutical products. However, in light of all the above limitations that characterize these devices, the dry powder inhalers are considered to be not only an alternative but also an innovative way to overcome these obstacles.

As far as the dry powder inhalers (DPIs) are concerned, two important stimuli increased the interest in such a delivery system. First, as has been already mentioned above, the Montreal Protocol in 1987, which banned the use of CFCs propellants was the milestone for the replacement of CFC-driven MDIs. Secondly, a recent stimulus came from concerns which arose regarding the use of nebulizers for severe acute respiratory syndromes as they could worsen their transmission (Frijlink and De Boer, 2004). As can be easily understood by their name, DPIs comprise devices in which the formulation is in the form of a dry powder (Sahane et al., 2012). Apart from the fact that they eliminate the need for use of propellants, they also present a variety of advantages which render them a substantial advancement in inhalation technology. First of all, since DPIs are activated by the patient's inspiratory airflow, they require minimum coordination between breathing and actuation of the device, providing a higher deposition within the lungs (Dal Negro, 2015). The minimal extrapulmonary deposition can be also rendered to the low device retention and the low exhaled dose. In addition, the fact that they are typically formulated as one-phase, solid particle blends, gives them a superior-unique chemical stability and bacterial growth resistance compared to solution formulations. Last but not least, thanks to their portability, patient friendly nature and ease of use, they provide patients' compliance and adherence to the therapeutic regimen (Sahane et al., 2012; Hadiwinoto et al., 2018).

2.3 Dry Powder Inhalers

The dry powder aerosols are produced by the dispersion of appropriately developed, metered quantity of powder in a stream of air generated by the patient's own inhalation. This is the reason why they are called dry powder inhalers (Emeryk et al., 2018).

The ideal DPI should present a number of characteristics that ensure its reliability, clinical efficacy and patient acceptance. These include:

- A device which is simple to use, portable, able to contain multiple doses and protect the formulation from moisture

- Accuracy and uniformity of delivered dose over a wide range of inspiratory flow rates
- Consistency of dose delivery throughout the lifecycle of the inhaler
- Appropriate particle size of the included formulation for deep lung deposition
- Minimum adhesion between drug formulation and device
- Cost-effectiveness
- Ideally, an incorporated mechanism to inform the patient about the dose administration (Islam and Gladki, 2008).

2.3.1 Classification of DPI devices

The main parts are the same for all types of the already marketed devices or even for those in development and are presented below.

- ❖ An adaptor to direct the device into the patient's mouth (mouthpiece)
- ❖ An aerolization mechanism
- ❖ A dose metering mechanism
- ❖ A deaggregation mechanism (Yadav and Lohani, 2013).

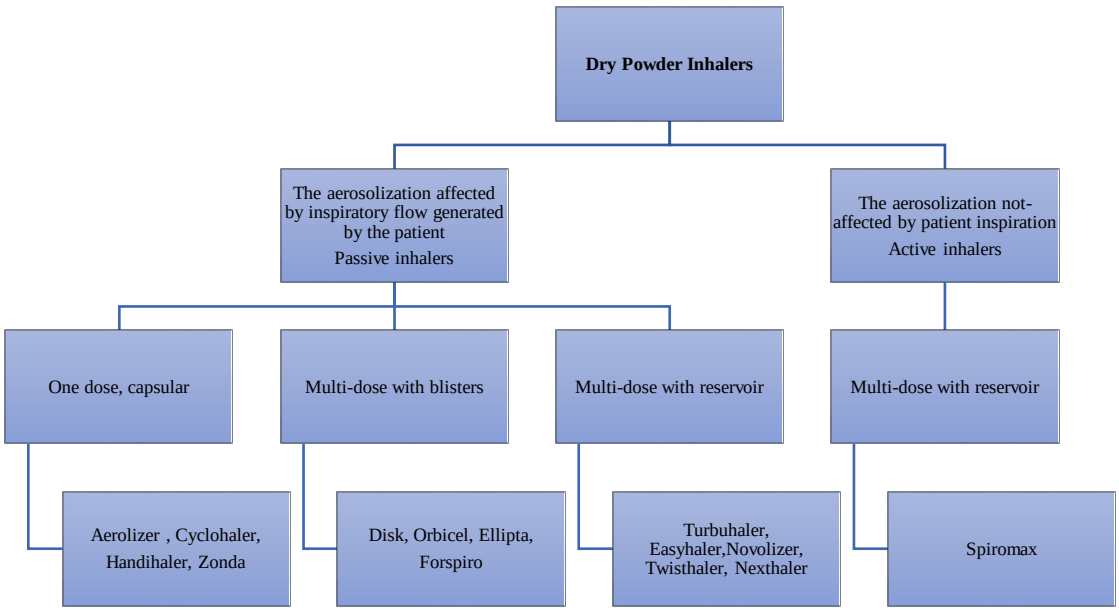


Figure 2.3 Types of dry powder inhalers (Emeryk et al., 2018).

DPI devices can be classified into classes according to the number of doses they can carry, the mechanism used for powder aerosolization and the intrinsic resistance they exhibit (Figure 2.3).

1. Dry powder inhaler devices can be classified based on the number of doses they can carry into:

    - ✓ Single-unit dose devices
    - ✓ Multi-dose reservoir type devices
    - ✓ Multi-unit dose devices

In a single-unit dose device, the formulation comprises of a micronized drug powder and usually a carrier system, supplied in individual capsules, which are inserted into the inhaler for a single dose administration, then removed and discarded after use.
As far as the multi-dose devices is concerned, they can be divided into two types: the reservoir type devices and multi-unit dose devices (Swain et al., 2012).

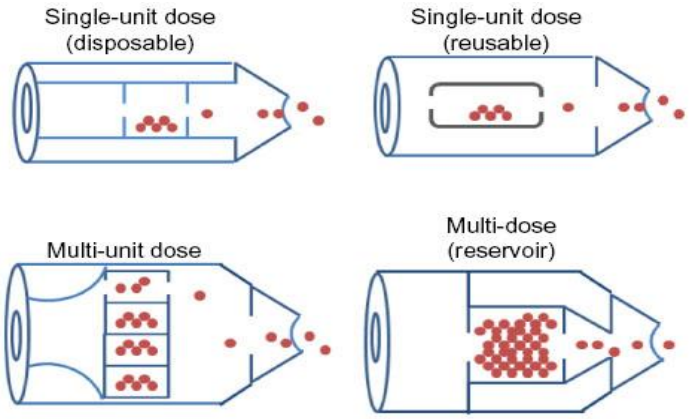


Figure 2.4 Illustration of four dose design options available for dry powder inhalers (Ibrahim et al., 2015).

Multi-unit dose DPIs use factory metered and sealed doses packaged in a manner that the device can hold multiple doses at the same time without requiring reloading. More specifically, the packaging may consist of replaceable disks, cartridges or strips of foil-polymer blister packaging that may or may not be reloadable. The advantage that characterizes this type of device could be the protection of pre-packaged doses from the environment until use (Daniher and Zhu, 2008).

On the other hand, multi-dose reservoir DPIs store the formulation in bulk and have an integrated mechanism for metering individual doses from the bulk after actuation. An important shortcoming regarding this type of device is the possible moisture ingress into the reservoir from the patient's exhalation or even the environmental humidity during the product's lifetime.

2. Based on the mechanism used for powder aerosolization, DPI devices can be divided into:

    - ✓ Passive devices
    - ✓ Active devices

The passive devices are breath-actuated and fully dependent on the patient's inspiration airflow, thus avoiding coordinating issues associated with pMDIs. The main defects of passive devices are the lack of uniformity concerning the inspiration force either among patients of different age and severity of disease, or even in the same patient (Ibrahim et al., 2015).
The concerns about the patients' ability to generate a sufficient flow rate for the appropriate powder dispersion resulted in the development of active (power assisted) DPI devices. These inhalers are designed with an integrated energy source, such as a battery-operated propeller or pressurized air from a hand piston, for the aerosolization of the powder bed in the DPI. However, such devices are not successfully marketed yet due to their complexity and thus user-unfriendly nature and high cost (de Boer et al., 2017).

3. Based on the intrinsic resistance exhibited by the device

Moreover, DPIs can be differentiated according to their intrinsic resistive regimen, which mainly depends on the design of each device. More specifically, there are the classes of the higher-resistance, medium-resistance and low-resistance devices. This type of classification affects the turbulence produced by the device, which combined with the inspiratory flow generated by the patient, determine the performance of each DPI (Dal Negro, 2015). Devices which operate at low inspiratory flow rate (higher-resistance) are favorable for children or even adults with decreased lung function, whereas the low-resistance device require a higher inspiratory flow rate generated by the patient (Yadav and Lohani, 2013). It is deeply understood that the balance between these two forces (the resistance of the device and the airflow generated by the patient) comprises a critical factor which determines the effectiveness of the DPI. The

higher the airflow, the higher the powder dispersion resulting in the generation of fine particles, even though usually the extremely high flow rate leads to oropharynx losses. On the other hand, a lower airflow favors the deeper lung deposition of the dispersed powder, even if a too low airflow can limit deposition inducing powder disaggregation and dispersion (Dal Negro, 2015).

2.3.2 Formulation design

Of critical importance in the development of powder inhalation products is the optimization and control of flow and dispersion (deaggregation) characteristics of the formulation. These properties are a function of various adhesive forces generated between particles, including Van der Waals forces, electrostatic forces and the surface tension of absorbed liquid layers (Figure 2.5) (Prime et al., 1997).

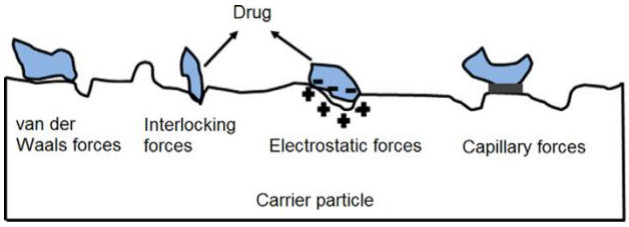


Figure 2.5 Physical interparticulate forces (Peng et al., 2016).

These interparticulate forces are dominant in the micronized powders that are required for inhalation therapy, (aerodynamic size < 5 μm) which also exhibit poor flowability properties due to the high surface free energy which makes them stick to each other (via cohesive forces) or to any surfaces they encounter (via adhesive forces) (Parsian et al., 2014). Among the different approaches concerning the DPI formulations are the formulation either of loose agglomerates of micronized drug particles with the required aerodynamic size or carrier-based interactive mixtures with micronized drug particles adhered onto the surface of coarse carriers, typically lactose (Figure 2.6) (Islam and Gladki, 2008).

Carrier-free formulations

The active pharmaceutical ingredient (API) can be in the form of a single compound, multi-compound composite or encapsulated particles. Various production techniques have been proposed for such formulations such as crystallization, milling, spray drying and supercritical fluid processing (Daniher and Zhu, 2008).

Drug-carrier formulations

The resulting poor flowability and aerosolization performance, most of the times, render the integration of appropriate carriers as flow conditioners crucial. Furthermore, the powder handling and the accurate dose metering for the development of formulations which contain only a low dose of the API becomes challenging (Peng et al., 2016).

As has been already pointed out above, the integration of larger coarse carrier particles is usually considered to be the adequate strategy for overcoming the limited intrinsic flowability properties and cohesiveness of APIs which are intensified in the micron size (1-5 μm) (Young et al., 2005). The functions of carriers can be summarized as follows:

- ✓ Improving the flowability of API particles to facilitate the filling (in blisters or capsules) during the development
- ✓ Increasing the dispersion of drug particles during emission (leucine has been used as dispersibility enhancer)
- ✓ Diluting the drug to allow metering of small quantities of potent drugs and thus ameliorate the accuracy and consistency of delivered dose (Peng et al., 2016)
- ✓ Some of the carriers used, such as lactose, with particular taste can assure the patient that a dose has been taken (Prime et al., 1997).

The geometric size of these carrier particles can range from 50 to 100 μm (Larhrib et al., 1999). The carrier of choice in DPI products, approved and generally accepted as safe by the regulatory agencies, is lactose. α-lactose monohydrate is the most commonly used carrier thanks to its desirable toxicological profile, physicochemical stability, broad availability, compatibility with the majority of low molecular weight drugs and relatively low cost (Hassan and Lau, 2009). Nevertheless, its bovine origin associated with the fear of the Transmissible Spongiform Encephalopathy (TSE) created the foundations for the development of new carrier options. The carriers that have been suggested as possible alternatives to lactose, are mannitol, sucrose, sorbitol, glucose or even cyclodextrine, raffinose, trehalose and xylitol (Hooton et al., 2006; Tee et al., 2000; Tang et al., 2006; Steckel and Bolzen, 2004).

One of the major drawbacks of the carrier-drug systems is the possible incomplete detachment of drug particles from the carrier particles' surface, poor redispersion of the interactive mixture upon device actuation, resulting in higher oral deposition, as the carrier particles are larger in size. Several parameters have to been taken into account when developing a drug-carrier

system for pulmonary drug delivery, whereas the most critical of them are the carrier payload (%w/w drug concentration in the mixture) and drug-to-carrier ratio (Daniher and Zhu, 2008).

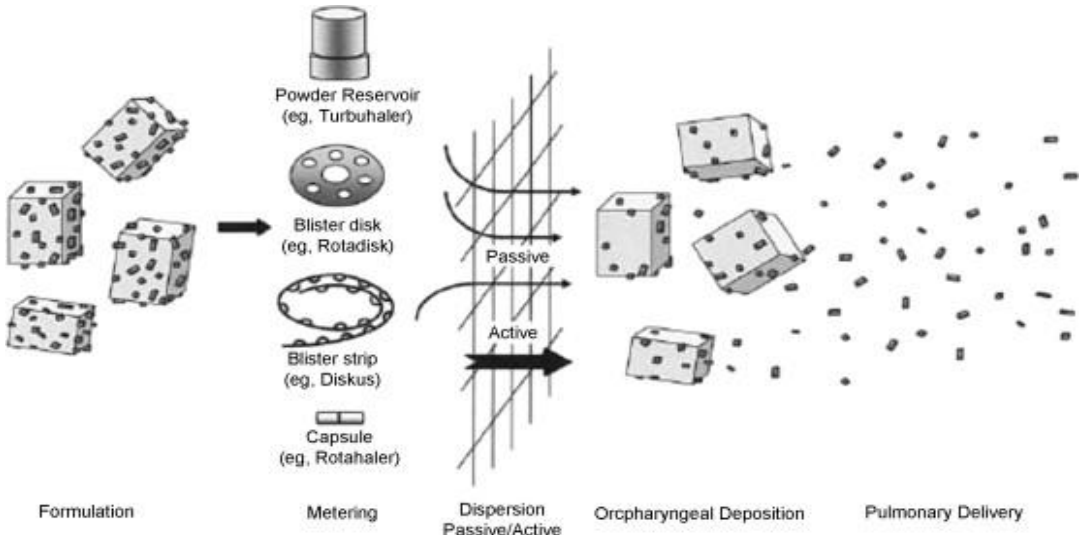


Figure 2.6 Principle of DPI design: blending of API and coarse carrier and deaggregation with inspiratory flow (Peng et al., 2016).

There are two approaches to produce such a DPI formulation. The first one is to dissolve the drug and the carrier in an appropriate solvent and then remove the solvent by spray drying or other methods. The second approach is to combine drug and carrier via particle interaction (Figure 2.7) (Daniher and Zhu, 2008).

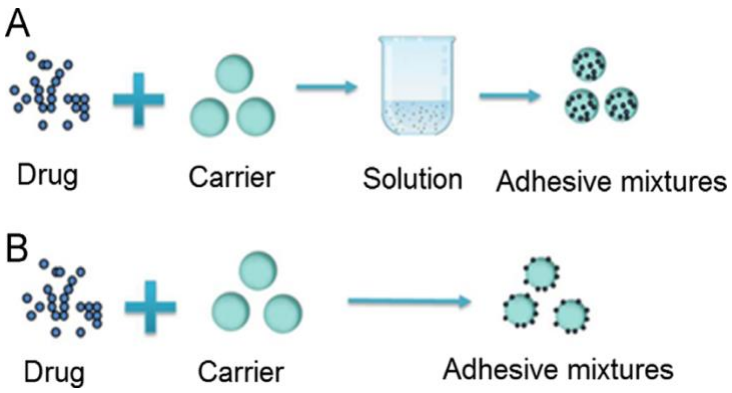


Figure 2.7 Two approaches for the drug and carrier combination in dry powder inhalers (Peng et al., 2016).

2.3.3 Mechanisms of aerosol generation and deposition

The efficiency of a dry powder inhaler (DPI) depends on the ability of drug particles to overcome the interparticle forces and to be dispersed into an aerosol during inhalation. The mechanism of aerosol generation has been described as a complex phenomenon. The aerosolization process can be roughly divided into four consecutive phases (Figure 2.8):

- Detachment from the static powder bed
- Fluidization
- Entrainment
- Drug resuspension (Hickey, 2003).

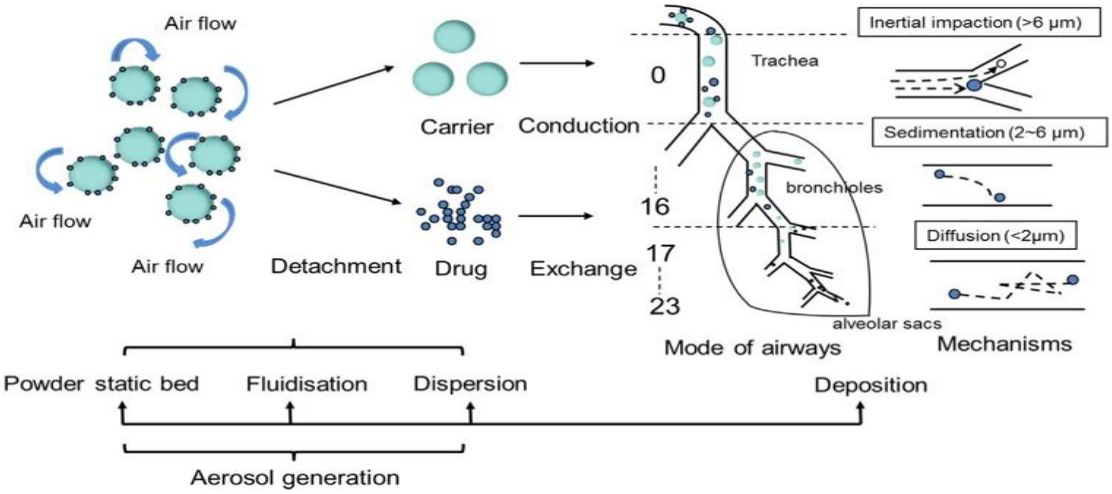


Figure 2.8 Mechanisms of aerosol generation and deposition (Peng et al., 2016).

More specifically, the airflow generated in DPI device during inhalation results in the transfer of kinetic energy into the initially static powder bed of the formulation. This procedure leads to the powder deaggregation. Powder fluidization is the process in which the powder mass, disturbed by the airstream, exhibits fluid-like properties. Following fluidization, the powder is entrained into the airflow. These two processes are critical and depend on the ability of a DPI to generate a therapeutically effective aerosol. Drug resuspension is mainly performed by deagglomeration forces including turbulent, inertial and impacting stresses and is followed by deposition of the drug in the respiratory tract (Peng et al., 2016).

Concerning the particle deposition in lung airways, there are typically three mechanisms, each of which is highly influenced by either the particle size or the anatomical properties and consequently the air velocity modifications lengthwise the respiratory tract:

- ✓ Inertial impaction
- ✓ Gravitational sedimentation
- ✓ Diffusion (Peng et al., 2016).

Impaction is the inertial deposition of a particle onto an airway surface and mainly occurs in the upper respiratory tract and oropharyngeal cavity, where flow velocities are high. As a result, the probability of impaction increases with increasing air velocity, rate of breathing, particle size (aerodynamic diameter [d_A] >5-6 μm) and density. Such particles are subsequently eliminated from the airways by swallowing or spitting (Hadiwinoto et al., 2018). Gravitational sedimentation (sedimentation under gravity) is the principal deposition that concerns particles over 0,5-2 μm and below 5-6 μm in size and occurs in the small conducting airways where the air velocity is lower. Slow inhalation is generally preferred to minimize inertial impaction in upper airways and to increase penetration into the lungs of large particles, whereas small particles are much less sensitive to fast/slow inhalation maneuvers. A breath hold, that is recommended during the DPI's use, gives time to particles to penetrate deep into the lungs to sediment on airway surfaces (Loira-Pastoriza et al., 2014). Ultrafine submicron-sized particles (aerodynamic diameter [d_A] <0,5 μm) acquire a random motion caused by the impact of surrounding air molecules. This named Brownian motion may result in particle deposition by diffusion, especially in small airways and alveoli, where airflow is very low (Pilcer and Amighi, 2010). Nevertheless, the normal human breathing does not provide the sufficient time for significant deposition of this size particles after a bolus dose. As a result, these nanoparticles are believed to be exhaled (Hadiwinoto et al., 2018). In summary, in order to reach the lower respiratory tract and optimize the pulmonary drug deposition, the optimal aerodynamic diameter should be between 0,5-1 μm and 3,5-5 μm (Pilcer and Amighi, 2010; Giovagnoli et al., 2017a).

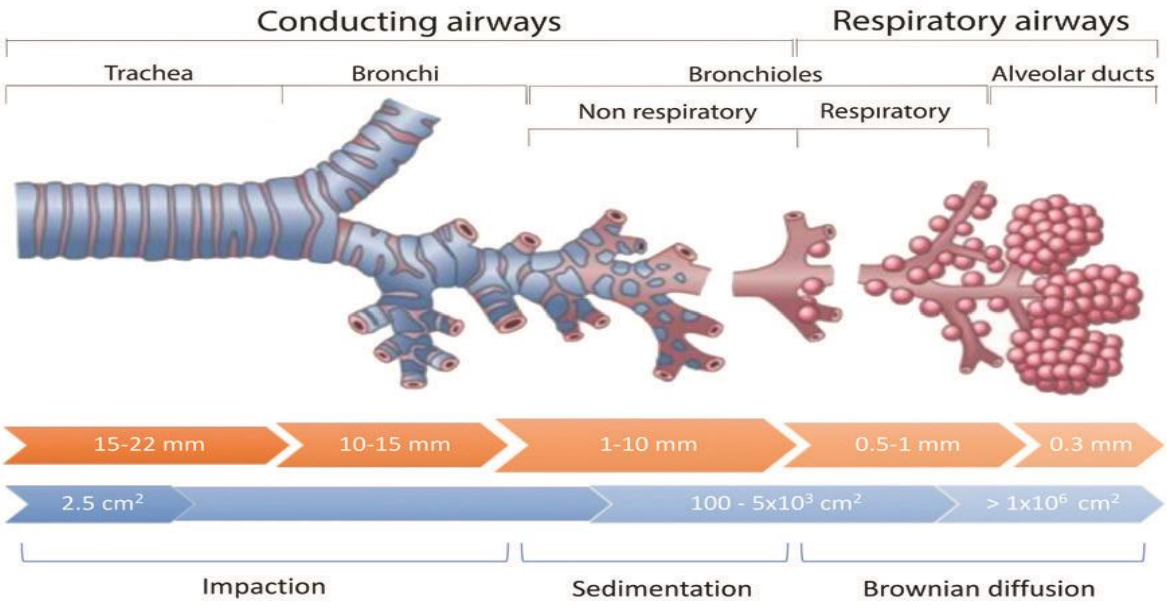


Figure 2.9 Mechanisms of inhalable particle deposition (Giovagnoli et al., 2017a).

2.3.4 Requirements of DPI formulations

The efficient delivery of a dry powder to the desired site of action in the lungs is crucial. The effectiveness of pulmonary drug to deposit at the desired site is affected by several factors, among which the physicochemical properties of drug particles are important (Hadiwinoto et al., 2018). Given that the number of excipients approved for use in pulmonary drug delivery for improving the aerosol behavior of formulations is restricted, ameliorated delivery efficiency may be achieved by developing optimized particulate formulations. Particle engineering stands for the controlled production of tailor-made inhalable drug particles which can be in pure physical form or combined with carriers as composite materials, of optimized size, morphology and structure. The critical quality attributes (CQAs) of the particle engineering process can be considered as the size, particle-size distribution, dispersibility (expressed as Emitted Dose ED Fine Particle Dose FPD, Fine Particle Fraction FPF), drug stability, bioavailability, specific targeting or even sustained release, always taking into account the specifics of inhaler design and drug delivery requirements. Therefore, the desired product characteristics include high FPF and ED, high dose consistency and uniformity, all of them independent by the inhalation flow rate. Apart from the desired aerodynamic diameter (expressed as the mass median aerodynamic diameter, MMAD), the optimized inhalable particles should exhibit the narrowest possible particle size distribution (PSD) (Pilcer and Amighi, 2010; Chow et al., 2007).

Since the particle size is known to influence the deposition in the respiratory tract, it is the most important design variable in an aerosol formulation, along with shape, density, electrical charge and hygroscopicity. The particle size can be expressed as the geometric diameter, measured by microscopic image analysis. However, the aerodynamic diameter is considered to be a more appropriate indicator of aerosol performance, since it reflects the real aerodynamic behavior and size of the dispersed inhaled particles. As pointed out above, the ideal aerodynamic diameter of particles included in DPI formulations is approximately 1-5 μm (Parsian et al., 2014). The aerodynamic diameter is defined as the diameter of a sphere of unit density that reaches the same velocity in airstream as the particle of interest, a non-spherical particle of arbitrary density (DeCarlo et al., 2004). The d_A is dependent on the geometric diameter (d_G), particle density (ρ) and the dynamic shape factor (χ), which is expressed by the following equation:

$$d_A \cong d_G \sqrt{\rho/\chi\rho_0} \qquad \text{[Equation 1]}$$

where ρ_0 is the unit density of spherical calibration spheres (Chow et al., 2007).

Taking the above equation into consideration, the aerodynamic diameter can be reduced by one or more of the following approaches:

- Decreasing the geometric diameter
- Reducing the particle density
- Increasing the particle dynamic shape factor

The shape factor is used to describe the ratio of particle drag force to that of a sphere of equivalent volume. The dynamic shape factor is affected by other particle properties such as the shape, surface roughness and surface area and as shown at the equation 1, it is inversely proportional to the d_A. Theoretically, a smaller d_A can be obtained with particles of non-spherical shapes, such as platelets, rods or fibers, because the χ value for such particles can be as high as 10. It has been observed that non spherical particles with high surface roughness have a greater drag force and thus smaller d_A compared to spherical particles with smooth surface (Tang et al., 2004). Moreover, as the particle density is proportional to the aerodynamic diameter, the lower it is, the smaller is the d_A (Parsian et al., 2014). The use of low-density powders that consist of porous particles has been proven useful to support efficient drug delivery (Hickey and Edwards, 2018). Last but not least, the particle shape has shown to play an important role at DPIs' performance. However, the use of shape factor is not sufficient to reflect the effect of particle shape on the particle aerodynamic behavior and thus the particle

orientation and contact area with other particles have to been taken into account as well. A study carried out in order to demonstrate the shape effect upon the particles' aerodynamic behavior proposed the pollen-shaped particles as an alternative approach to spherical particles that have been studied, since the first ones exhibited better flowability, higher emitted dose, and higher fine particle fraction than particles of other shapes in similar size range (Hassan and Lau, 2009).

2.3.5 Techniques for the production of engineered particles

The principle quality attributes of the manufacturing process of dry powders for inhalation are the particle size, the particle size distribution and the appropriate formation of solid-state particles. Crystallization or direct-controlled crystallization may result in the production of particles of appropriate properties for pulmonary drug delivery, though in some cases may not be feasible due to economic or technical reasons. Hence, size reduction through micronization is often used in pharmaceutical industry either in the suspended state (wet milling) or in the dry state (jet milling). Nevertheless, advanced techniques have been also used in order to incorporate the particle formation and drying in only one step (Hadiwinoto et al., 2018).

- Direct-controlled crystallization

Solution crystallization is the formation of a crystallized solid from a solution. The main purpose is the fabrication of particles of desired properties in a single step through control over the crystallization kinetics, a procedure called direct-controlled crystallization. The driving force for crystallization is supersaturation. More specifically, controlled crystallization of hydrophobic drugs in the respirable size range can be achieved by an anti-solvent precipitation technique using growth-retarding stabilizing additives such as hydroxylpropylmethylcellulose (HPMC). The obtained precipitated drug crystals exhibit higher FPF values (Chow et al., 2007).

- Spray drying technique

Spray drying is an advanced pharmaceutical manufacturing process used to produce respirable particles in solid state (Mosén et al., 2005). The spray-drying technique is based on the transformation of a liquid into a dry powder by atomization in a hot drying gas stream, typically air. The spray-drying process consists of four principal steps: atomization of the liquid feed into a spray, spray-air contact, drying of sprayed droplets and formation of dry particles, separation and collection of the dry product from the drying gas (Figure 2.10). The fluid is fed into the drying chamber by a peristaltic pump through an atomizer which can be a rotary atomizer, a pressure nozzle or a two-fluid nozzle. The droplets generated by the atomization are subjected to fast solvent evaporation resulting in the formation of dry particles, which are then separated from the drying gas by means of a bag filter, cyclone or electrostatic precipitator. The feed liquid can be a solution, suspension, emulsion or even paste (Sosnik and Seremeta, 2015). Among the variety of advantages that this method presents are the ease of atomization and rapidity (one-step process), which facilitate the process standardization, thus reducing the variability and improving the technology transfer to large-scale production (Giovagnoli et al., 2014a). Furthermore, another remarkable advantage of spray-drying is the possibility to dry a broad spectrum of compounds, including the heat-sensitive ones-such as proteins and peptides-thanks to the fast solvent evaporation from the surface of produced small droplets, which exhibit high surface area-to-volume ratio (Sosnik and Seremeta, 2015). Last but not least, spray drying is advantageous in terms of producing flowable powders with a controlled particle size distribution (Ishwarya et al., 2015).

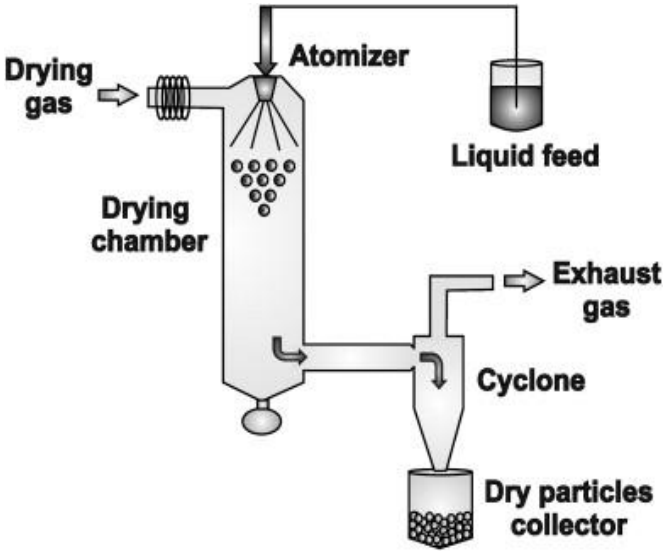


Figure 2.10 Illustration of spray-drying technique

- Spray freeze drying method

It is an advanced particle engineering method which combines the principles of spray-drying and freeze-drying methods. Spray freeze drying (SFD) is a three-step process which involves the atomization of a liquid into droplets, the solidification with a cold fluid (typically liquid nitrogen) and then sublimation at low temperature and pressure. The possibility of encapsulating low water -soluble drugs and the unique aerodynamic performance of the porous particles produced, render the SFD an attractive process for producing respirable particles. Nevertheless, the complexity and high cost of the process limits its use (Ishwarya et al., 2015).

- Supercritical fluid technology

Supercritical fluid (SCF) technology comprises an interesting approach for particle formation. The SCF process is based on the principle of solution enhanced dispersion by supercritical fluid (Rehman et al., 2004). It provides micro- or even nanoparticles with narrow particle size distribution and can be also used for microencapsulation. It is a single-step process, environmentally acceptable which can give high porosity particles, the possibility of processing thermolabile molecules and can provide control of crystal polymorphism (Fages et al., 2004).

2.4 Limitations-Challenges associated with dry powder inhalers

In light of all the above considerations, it is undeniable that a challenging element that has to been taken into account for the development of new DPIs or even the improvement of those being already marketed, is the balance that has to been achieved between the three principal types of forces that determine the effectiveness of pulmonary drug delivery: the inteparticulate forces in the powder formulation, the dispersion forces generated by the inhaler and the deposition forces onto the surfaces of the respiratory tract (Figure 2.11). The formulation, the device and the patient are the three important players which have to been taken into account since they influence the overall clinical performance of DPIs. However, the efficient strategy is not controlling and evaluating these parameters separately but taking into consideration their interactions. A characteristic example that can be considered as strong evidence for such an interaction is the first inhaled insulin, Exubera ® DPI, which was withdrawn one year after its approval because of poor patient compliance due to the cost and bulkiness of the device (Price et al. 2018). The co-operability of formulation and device is the challenge that has to be attained

when developing a DPI product and which renders DPIs more complex compared to conventional dosage forms (Buttini et al., 2018).

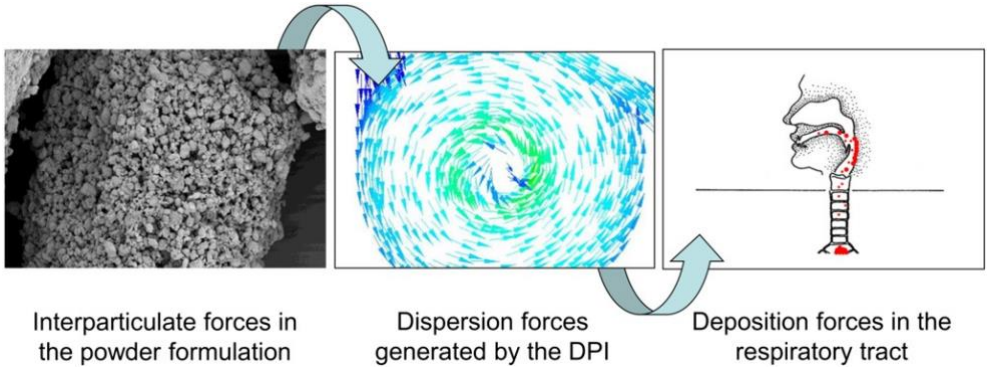


Figure 2.11 Desired balance for obtaining an optimal DPI therapy (Hoppentocht et al., 2014).

DPIs for high dose drugs continuously become an interesting and at the same time challenging approach. Such drugs are the antibiotics for which, as has been mentioned above, the pulmonary route of administration is a promising alternative. However, the poor flowability properties that typify the majority of the drug substances are intensified when the dose is higher in the formulation and in these cases, the implementation of particle engineering becomes crucial (Hoppentocht et al., 2014). More specifically, such formulations require the development of powders which are carrier-free or contain small amounts of excipients. Hence, the use of excipients in high dose drug formulations is restricted due to the inevitable dilution of the drug and thus the requirement of increasing the powder mass. Despite the fact that such powders can be easily produced, their dispersibility properties are restricted by the high cohesiveness that they exhibit (Eedara et al., 2018).

As has been already mentioned above, the fine particles required for DPI formulations which present poor flowability properties, hinder not only the homogeneity of produced mixtures but also the filling of blisters or capsules. Consequently, the DPI manufacturing process exhibits high variability for a given attribute and thus low capability, rendering the implementation of quality by design (QbD) principles crucial, within the context of profound process knowledge needed. It is undeniable that such a multiparametric DPI development can be successfully transacted only through QbD-driven statistically designed experiments (Buttini et al., 2018). Another important shortcoming concerning the dry powder inhalers and generally the pulmonary drug delivery is the incapability to control the pharmacokinetics of inhaled drugs beyond a few hours. The rapid absorption of drug substances or even the efficient clearance from the deep lung through phagocytosis in the alveolar region, render the sustained drug

release pulmonary administration important (Callard Preedy and Prokopovich, 2013). However, at present there are no SR (sustained release) products on the worldwide market for pulmonary drug delivery in spite of the active research performed in this field (Cook et al., 2005).

Moreover, patient's education concerning the adequate use of the device is a highly influential parameter. It has been reported that handling errors concerning the inhaler device are underestimated. In fact, studies have demonstrated that the rate of incorrect use of DPI devices ranges from 10 to 55%, whereas the type of failure is dependent on the type of device (Aydemir, 2015).

Last but not least, the restricted spectrum of FDA approved excipients for pulmonary administration limits the development of novel formulation strategies (Table 1). The introduction of new excipients for inhalation requires complete toxicological evaluation and in vivo studies of the whole formulation (Pilcer and Amighi, 2010; Edge et al., 2008).

EXCIPIENTS	ROLE	CURRENT STATUS
lactose	carrier	**Approved**, carrier of choice
glucose	carrier	**Approved**
mannitol	carrier	**Approved**
trehalose	carrier	Promising alternative
Magnesium stearate	Protection from moisture	**Approved**
DPPC, DSPC, DMPC, cholesterol	Used in liposomes	Biodegradable Biocompatible excipients
Leucine	Improvement of aerosol efficiency	Endogenous substance
Poloxamer	Production of light and porous particles	May not be pro-inflammatory at low dose
Bile salts: DCA	Absorption enhancer, increase of retention time in the lungs	Endogenous substances. May be accepted at low doses
Hydrohypropylated-β-CD	Absorption enhancer	Promising
Chitosan	Absorption enhancer	Pro-inflammatory effects observed
PLGA, PLA	Used in sustained release formulations	Immunogenicity effect observed

Table 2.1 List of accepted or interesting excipients for DPI formulations (Pilcer and Amighi, 2010; Edge et al., 2008).

3. AIMS

- ✓ **To formulate an innovative dual release DPI formulation containing an antibiotic (capreomycin) complex and an anti-inflammatory drug for potential use in pulmonary infections,**
- ✓ **to determine suitable conditions for the preparation of homogeneous microparticle (MP) formulation blends by spray drying comprising glidant MP and biodegradable polymeric MP and**
- ✓ **to evaluate the aerodynamic, morphological properties as well as the drug release profile from the above proposed blend.**

4. MATERIALS AND METHODS

4.1 Drug substances selected

4.1.1 Model Drug A/ Capreomycin sulfate

Capreomycin is an approved second-line antitubercular drug first described in 1960 by Herr and his associates at the Lilly Research Laboratories (Donomae, 1966). It is isolated from the fermentation of *Streptomyces capreolus* and its therapeutic effect is expressed by the inhibition of bacterial growth by blocking the protein synthesis on the ribosome (Herr and Redstone, 1966; Johansen et al., 2006). Capreomycin is a cyclic pentapeptide which exists as a mixture of four microbiologically active compounds, namely I_A, I_B, II_A, II_B with the following proportion: 90% I_A and I_B forms and 10% II_A, II_B) (Garcia-Contreras et al., 2007; Nomoto et al., 1977). It is used intramuscularly or intravenously, since the oral administration is restricted by its protein nature, in combination with other potent drugs for the treatment of tuberculosis in cases that the first-line drugs are inefficient and is indicated also for multiple drug-resistant tuberculosis (MDR-TB) (Ricci et al., 2006). In general terms, the second-line antitubercular drugs are characterized by low incidence of resistance, whereas their high toxicity- capreomycin is notorious for nephrotoxicity and ototoxicity- increases the interest for the development of novel delivery systems (Bastian and Colebunders, 1999). More specifically, capreomycin respirable powder has been evaluated in clinical trials (Phase I) with interesting results (Schoubben et al., 2013). Among its physicochemical properties, the most important is the high water-solubility, resulting in rapid absorption into the blood stream, a phenomenon which requires further formulation strategies (Giovagnoli et al., 2014b). The capreomycin powder used for the current study was purchased by Sigma-Aldrich Co., Italy.

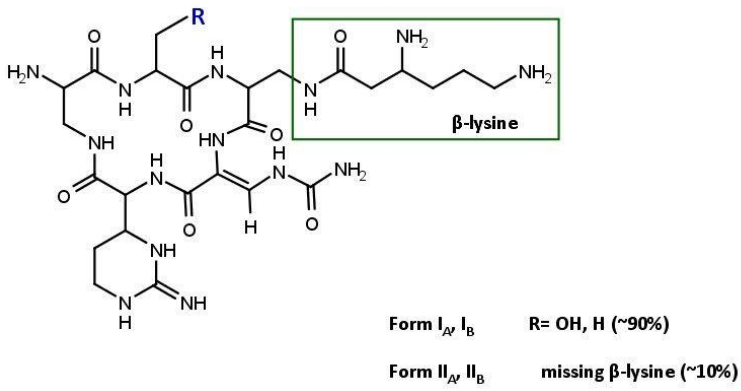


Figure 4.1 Capreomycin chemical structure (designed by MarvinSketch version 17.9).

4.1.2 Model Drug B

Model drug B belongs to the nonsteroidal anti-inflammatory drugs (NSAIDs). These agents decrease the symptoms of inflammation and exhibit a wide range of pharmacological activities, such as analgesic, antipyretic and antiplatelet.

4.2 Excipients selected

4.2.1 Deoxycholic acid (DCA)

Deoxycholic acid is a secondary bile acid, naturally formed by bacterial 7-dehydroxylation of cholic acid in the colon and it comprises the 12-26 % of biliary bile acids (Hofmann et al., 2018). In the current study, sodium deoxycholate (Sigma-Aldrich Co., Italy) was used for the physical modification of the model drug A-capreomycin sulfate with the aim to develop a more hydrophobic complex, given the high water-solubility that capreomycin exhibits, which results in a high burst of release from pharmaceutical formulations. The enhanced hydrophobicity of such complexes which are formed spontaneously in water has been proposed as a strategy for the prolonged accumulation of drugs in the lungs and in case of tuberculosis for the increased intracellular penetration (Giovagnoli et al., 2014a; Giovagnoli et al., 2014b; Giovagnoli et al., 2017b).

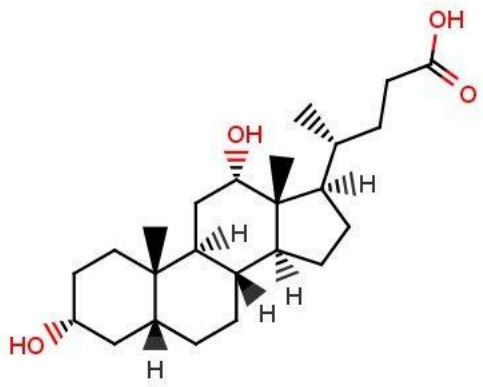


Figure 4.2 DCA chemical structure (designed by MarvinSketch version 17.9).

4.2.2 Polylactic acid (PLA)

Polylactic acid (PLA) belongs to the family of aliphatic polyesters, made from α-hydroxy acids. It is a thermoplastic, biodegradable polymer and has low toxicity. This degradation simply occurs by hydrolysis of the ester bond and results in production of lactic acid molecules, which are then broken down into water and carbon dioxide via the citric acid cycle. It has a glass-transition and melt temperature of about 55ºC and 175ºC respectively (Garlotta, 2001). In general, polylactic or poly(lactide-co-glycoside) (PLGA) polymers are approved by FDA only for parenteral administrations (Giovagnoli et al., 2017a; Fredenberg et al., 2011). However, the fact that they are biodegradable polymers has increased the interest for the development of advanced delivery systems for inhaled therapy, such as microparticles. Such PLA microparticles have been described and proposed at the literature for the fabrication of sustained release (SR) particles for pulmonary drug delivery (Baras et al., 2000; Muttil et al., 2007; Anish et al., 2014; Cook et al., 2005). SR drug delivery to the respiratory tract offers a variety of advantages, including extended duration of action, reduction of adverse effects and improved compliance to the therapeutic regimen (Cook et al., 2005). In the present study, the polymer used for the production of sustained release microparticles was a low-molecular weight PLA (Resomer ® R 202 H, Boehringer Ingelheim Pharma GmbH & Co.KG).

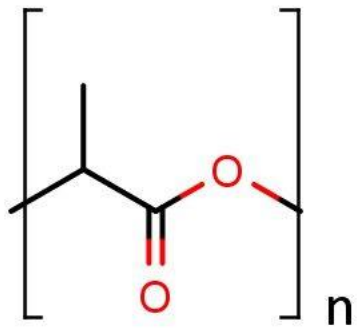


Figure 4.3 PLA chemical structure (designed by MarvinSketch version 17.9).

4.2.3 Leucine

L-leucine (LL) has been widely used in DPI formulations mainly as dispersibility enhancer as well as for moisture protection (Shur et al., 2008; Aquino et al., 2012; Feng et al., 2011; Seville et al., 2007; Boraey et al., 2013; Chang et al., 2014; Eedara et al., 2018). In the current study, L-leucine (Sigma-Aldrich, USA) had a dual role: as a carrier-drug delivery system with a faster drug release profile and as dispersibility enhancer for PLA microparticles which exhibit low aerodynamic performance. L-leucine has been characterized as a surface-active excipient, since it is believed to absorb at the droplets' surface during the spray-drying process and thus to reduce their contact area and the cohesiveness of the obtained powders, by modifying the surface morphology (Eedara et al., 2018).

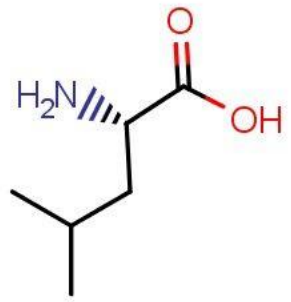


Figure 4.4 L-leucine chemical structure (designed by MarvinSketch version 17.9).

4.3 Methods used

4.3.1 The preparation of Capreomycin sulfate-DCA complex

The novel CS-DCA complex was first described at the laboratory of Pharmaceutical Technology at the University of Perugia, Italy. Based on previous studies carried out at this laboratory, was selected the ratio 1:4 Capreomycin: DCA, which gives the optimum yield of the reaction. The complex was prepared using water as a solvent. Briefly, appropriate amounts of capreomycin and DCA were first solubilized separately in 1 and 3 mL water respectively. After the complete solubilization of both materials, CS solution was added dropwise to the DCA solution under stirring in order to obtain the HIP (hydrophobic ion-pair). The obtained suspension was centrifugated at 4000 rpm for 20 minutes and after removing the supernatant, the CS-DCA complex was retained in the freezer. The obtained CS-DCA complex was then freeze-dried for 15 hours with the aim to remove any residuals of water.

4.3.2 Fabrication of Capreomycin-DCA complex and model drug B loaded *PLA* microparticles

The production of the PLA microparticles was carried out using the Mini Spray-Dryer, Buchi B-290 (Figure 4.5).

Preliminary solubility experiments demonstrated that the CS-DCA complex is soluble in the following organic solvents: DMSO, ethanol, methanol. Given that the polymer (PLA) is known to be soluble in acetonitrile, a mixture of methanol: acetonitrile (30:70% v/v) was used for the preparation of feed solution. CS complex and Model drug B were co-encapsulated into PLA microparticles (MPs) (CS complex/model drug B: polymer 20:80% w/w drug loading). The total polymer concentration used was 1% w/v. For the preparation of feed solution, 60 mg CS-DCA were dissolved in 8 mL methanol, whereas 60 mg model drug B and 240 mg polymer were dissolved in 16 mL acetonitrile.

Figure 4.5 Mini Spray-Dryer Buchi B-290 (https://www.buchi.com/en/products/spray-drying-and-encapsulation/mini-spray-dryer-b-290).

The process conditions are summarized below (Table 4.1):

Parameter	Value
Inlet temperature	78ºC
Air flow rate	357 L/h
Feed rate	3.2 mL/min
Aspirator capacity	20 m^3/h

Table 4.1 Mini Spray-Dryer B-290 conditions (Giovagnoli et al., 2014a).

4.3.3 Fabrication of Capreomycin-DCA complex and model drug B loaded *Leucine* microparticles

The production of Leucine microparticles was carried out using the Nano-Spray Dryer, Buchi B-90, which is a laboratory scale spray-dryer. The Nano Spray Dryer B-90 is based on a new spray drying concept (Figure 4.6). The drying gas enters the apparatus from the top in a laminar flow. It is heated up to the selected inlet temperature, flows through the drying chamber and is then filtered before exiting the instrument (outlet temperature). The feed solution is fed to the spray head through a peristaltic pump. The generation of fine droplets occurs thanks to a piezoelectric driven actuator (60 kHz) which vibrates a thin, perforated, stainless and steel membrane. The membrane (spray mesh) used exhibited micron-sized holes (5.5 μm). The fine droplets are then dried into solid particles which are collected by electrostatic charging. Finally,

the powder was collected from the electrostatic precipitator using a rubber spatula (Li et al., 2010).

Given that leucine is known to be soluble in both ethanol and water, whereas CS-DCA complex was found to be soluble in ethanol, a mixture of ethanol: water (30:70% v/v) was used for the preparation of feed solution. CS complex and Model drug B were co-encapsulated into Leucine microparticles (MPs) (CS complex/model drug B: polymer 20:80% w/w drug loading). It was observed that the increased temperature (50 ^{o}C) contributes to the solubilization of CS complex in this mixture and for this reason the solution was kept in 50 ^{o}C during the spray-drying process in order to avoid any precipitation.

The process conditions are summarized below (Table 4.2):

Parameter	Value
Inlet temperature	100 ^{o}C
Air flow rate	95 L/min
Pump rate	100 %
Pressure	45 mbar

Table 4.2 Nano Spray-Dryer B-90 conditions

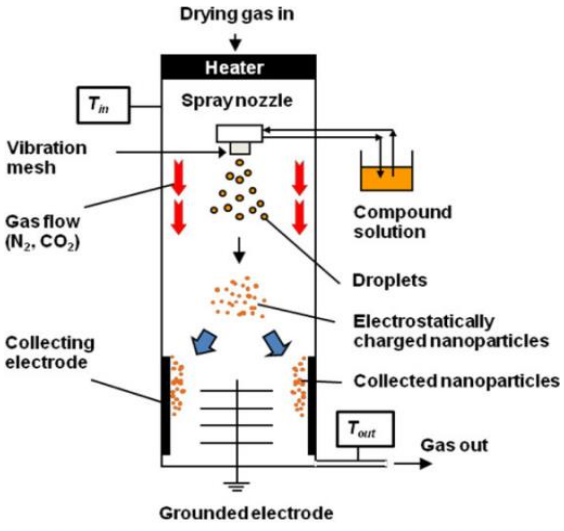


Figure 4.6 Principle of operation of the Nano Spray Dryer Buchi B-90 (Li et al., 2010).

4.3.4 Mixing of *PLA* and *Leucine* Microparticles

The mixing of spray-dried PLA and Leucine microparticles was carried out with a cochlea mixer (EUROSTAR IKA®-WERKE) (Figure 4.7). A 2^2 full factorial design study was undertaken to evaluate the effect of process variables on particle characteristics. The factors selected for the design were the mixing time with low level 5 minutes and high level 15 minutes and the PLA/leucine blend ratio, with low level 30/70 and high level 70/30 (Table 4.3). The rotation speed (40 rpm) and the temperature during the mixing processing (25 ^{o}C) were kept constant. Among the critical quality attributes (CQAs), such as fine particle fraction, content uniformity, emitted dose and Hausner ratio of obtained mixed microparticles, %Fine Particle Fraction (%FPF) was selected as response of the design.

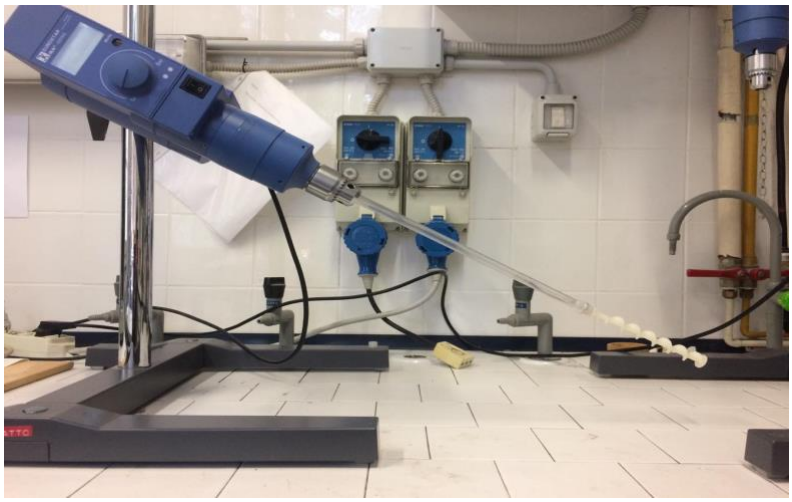


Figure 4.7 The cochlea mixer

Factors	Low level	High level
Mixing time (min) (A)	**-1**	**+1**
PLA/Leucine blend ratio (%w/w) (B)	**-1**	**+1**

Table 4.3 Factors and levels for the experimental design study

4.3.5 Selected analytical method

The analytic method selected for the quantification experiments and the characterization tests of microparticles was the HPLC (high performance liquid chromatography) method. The method selected was a RP-HPLC (reverse phase HPLC) method, inspired by the published work by Rossi et al. (Rossi et al., 2004). RP-HPLC analysis for the co-determination of both model drug A and B, was carried out using a Hewlett Packard HP 1050 Series chromatograph and a reverse-phase column (Luna C18, 5 μm, 150 mm x 4.6 mm) (Phenomenex, USA). The detector used was a UV-Vis detector (Hewlett Packard HP 1050 Series) and the wavelength was set up at 268 nm. Elution was carried out in an isocratic manner with a flow rate 1 mL/min using a mixture acetonitrile-H_3PO_4 buffer solution (pH 2.3; 0,009 M) with 0.3% heptafluorobutyric acid (84:16% v/v). The temperature of the column was stabilized at 25ºC. The calibration curves were carried out with five solutions in the concentration range of 1.25-15 μg/mL and 5-25 μg/mL for model drug A(CS) and model drug B, respectively (Figure 4.8, Figure 4.9). The data presented are the result of three measurements (n=3). Concerning the CS, the data were calculated as the sum of the CS I_A and I_B peaks. Concerning the quantitative determination of capreomycin complexed to DCA, appropriate amount of complex (≅ 5 mg) was dissolved in 10 mL HCL 1M. It was concluded that in acidic environment, the complex is separated and the precipitation of DCA is observed. For the determination of both drugs in PLA microparticles, appropriate amount (≅ 5 mg) was dissolved in 1 ml (50-50% v/v ethanol-acetonitrile) and was then diluted in HCL 1M and mobile phase before injected in the HPLC, whereas in leucine microparticles the solvent was modified to (50-50% v/v ethanol-water). All experiments were performed in triplicate.

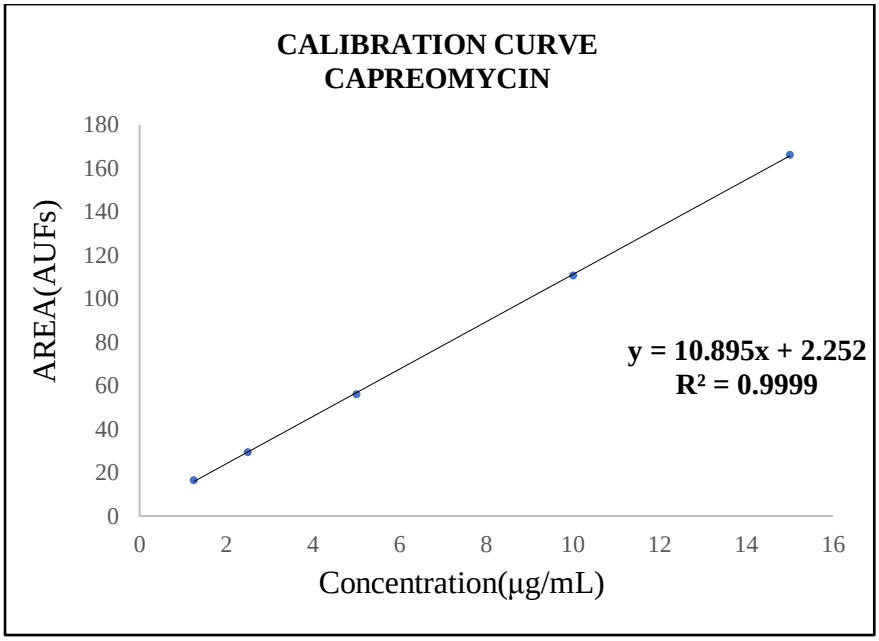


Figure 4.8 Capreomycin Calibration curve

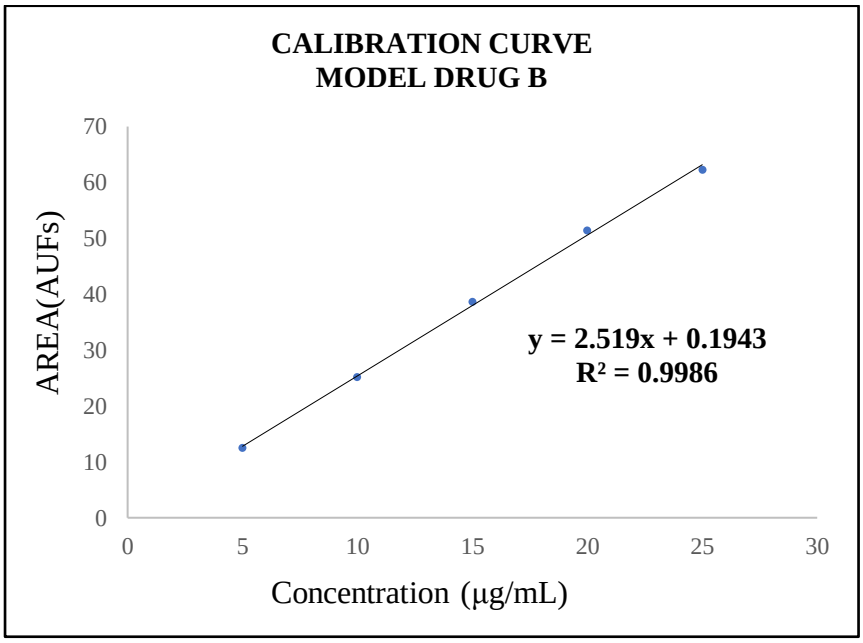


Figure 4.9 Model drug B Calibration curve

4.3.6 Glass twin-stage impinger

The regulatory requirements (FDA, EMA, ICH and others) include, among others, the in-vitro aerodynamic and dispersibility assessment of fine particles in DPIs and the evaluation of emitted dose (ED) and fine particle dose (FPD) in the context of the required quality and effectiveness of pharmaceutical products. The European Pharmacopeia (Ph. Eur.) proposes, among others, the use of Ph. Eur. Apparatus A glass Twin Impinger (Figure 4.10). The glass twin-stage impinger consists of two stages and is called Apparatus A due to its importance as a simple and inexpensive quality control (QC) tool. Despite the generally accepted opinion that an impactor should include at least five stages in order to express precisely the aerodynamic performance of the controlled powder, the twin stage impinger provides satisfying and sufficient results. The major advantage that characterizes the Twin stage instrument is the fact that it consists only of glass parts, which are not subjected to corrosion compared to the conventional metallic impactors. Its principle of operation is based on the division of the emitted dose from the controlled device into the respirable and non-respirable portion. More specifically, the non-respirable dose concerns the powder that deposits on the oropharynx and is then swallowed and this is considered to be the Stage 1. The respirable dose of the appropriate aerodynamic performance, which is able to penetrate into the lower respiratory tract is considered to be the Stage 2. Particles with aerodynamic size lower than 6.4 μm pass into the lower chamber of the impinger. The in vitro dispersibility assessment of the different mixtures of the design was carried out according to the procedure proposed by the European Pharmacopeia (Edition 9, Procedure for powder inhalers). Thus, prior to each test, 7 mL of appropriate solvent (10:90%v/v acetonitrile: water) were put into the upper impingement chamber and 30 mL of the same solvent into the lower impingement chamber. For the dispersibility assessment of PLA and leucine microparticles separately the solvent was modified. An appropriate amount of powder (≅20 mg) was added to an HPMC capsule (Type 3, Quali-V, Qaulicaps ® S.A.U, Spain). When the apparatus was correctly assembled and the air flow was set up at 60 ±5 liters /min, the pump was switched on for 5 seconds. After each twin-stage test, the collected powder was diluted (in total 50 mL stage 1; 100 mL stage 2; 2 mL in capsule; 10 mL in device) and was assayed by means of HPLC instrument. Each experiment was performed in triplicate, whereas for those with high observed variability, further repeats were conducted.

Figure 4.10 The twin stage impinger

4.3.7 Tapped density analysis

The tapped density (ρ_{tapped}) assessment was performed by ERWEKA tapped density tester, type SVM 102 (Heusenstamm, Germany). Without compacting, an appropriate amount of powder (≅200 mg) was introduced into a dry cylinder and the initial (unsettled apparent) volume, V_0 was measured. After carrying out 1250 taps, the final tapped volume was also measured. The Hausner ratio was calculated using measured values of bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}), as following:

$$Hausner\ ratio = (\rho_{tapped} / \rho_{bulk}) \qquad \text{[Equation 2]}$$

The flowability properties of powders were evaluated according to the scale of flowability (Eur. Pharmacopeia, Edition 9)

Hausner ratio	Flow character
1.00-1.11	Excellent
1.12-1.18	Good
1.19-1.25	Fair
1.26-1.34	Passable
1.35-1.45	Poor
1.46-1.59	Very poor
>1.60	Very, very poor

Table 4.4 Scale of flowability (Eur. Pharmacopeia, Edition 9)

4.3.8 Particle size analysis

Particle size analysis was carried out by an Accusizer C770 particle counter (PSS, Santa Barbara, California USA) equipped with an autodilution system (Figure 4.11). PLA microparticles were dispersed in 1% w/v Tween 80 solution, then slightly sonicated and analysed. Particle size of leucine microparticles was determined by Scanning electron microscopic (SEM) images, using Image J, due to their high solubility in most of the allowed solvents and water. A semi-manual particle counting over an average of 200 particles was performed over at least six different SEM microphotographs and size distributions and mean diameters were calculated.

Figure 4.11 Particle size analyzer

4.3.9 Scanning electron microscopy (SEM)

PLA, leucine microparticles as well as their blends were all morphologically characterized by scanning electron microscopy (SEM) by means of a FEG LEO 1525 microscope (LEO Electron Microscopy Inc., NY). The acceleration potential voltage was kept constant at 1 keV. Samples were firstly placed onto carbon tape coated aluminium stubs. Then, prior to the analysis, the stubs were coated with chromium by a high-resolution sputter (Quorum Technologies, East Essex, UK) for 20 s at 20 mA. Additional observations were conducted on nebulized blends in order to address particle behaviour upon emission from the device. To do so, emitted particles were collected from the impinger immediately after actuation.

4.3.10 Differential scanning calorimetry (DSC) analysis

Differential scanning calorimetry (DSC) analysis was performed using a Mettler Toledo DSC821e calorimeter (Mettler Toledo, Milan, Italy) calibrated using indium standards. Holed aluminum pans were employed in the experiments for all samples and an empty pan, prepared in the same way was used as a reference. Samples of 2-6 mg were weighted and thermal scans were conducted at a heating rate of 10^{o}C, from 25 to 200 ^{o}C. Data were evaluated with STARe software.

4.3.11 Drug release test -Dialysis method

For the evaluation of release profile of model drug A and model drug B from the PLA and Leucine microparticles, as well as their blend, the method selected was the regular dialysis using the dialysis bag (Figure 4.12). More specifically, an appropriate amount of powder (≅ 5mg) was introduced into the dialysis bag containing an amount of release media for the membrane activation (5 mL Phosphate Buffer 0.01M; pH 7.4), which was sealed and placed in a large falcon containing release media (30 mL Phosphate Buffer 0.01 M; pH 7.4). The falcons were retained at 37.4^{o}C. Thus, the drug released from the microparticles (the polymer is retained into the dialysis bag due to its larger size), diffuses through the dialysis membrane to the outer media (receptor compartment), where it is dissolved and sampled for analysis. The sample volume selected was 0.5 mL. Each experiment was performed in triplicate.

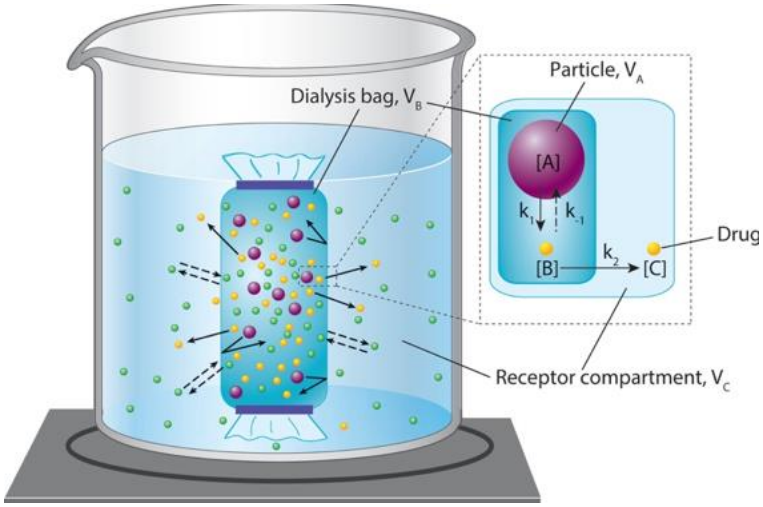


Figure 4.12 Drug diffusion through dialysis bag. Yellow spheres are drug molecules
[A] is the drug concentration in the particle, [B] the drug concentration in the inner media and [C] the drug concentration in the outer media (Jug et al., 2018).

5. RESULTS AND DISCUSSION

5.1 Capreomycin in capreomycin-DCA complex

The amount of capreomycin complexed to DCA was determined according to the method described above. The drug content was calculated according to the following equation:

{[C(μg/mL) x100]/1000}/ weight of complex, [Equation 3]

where C the concentration corresponded to the capreomycin calibration curve and weight of complex the precise amount of complex weighted for the quantitative determination of capreomycin.

Sample	Amount of Capreomycin(mg)	Average	±SD
1	0.4097	**0.4412**	**0.0273**
2	0.4588		
3	0.4550		

Table 5.1 Drug content of capreomycin in capreomycin-DCA complex

It is thus concluded that 44.12 % of capreomycin is complexed to deoxycholic acid for the capreomycin-DCA ionic complexation and compared to the theoretical amount of capreomycin available for complexation, the % binding efficiency is 100%.

5.2 Quantification of model drug A and model drug B in PLA microparticles

The amount of model drug A-capreomycin and model drug B encapsulated into the PLA microparticles was calculated according to the already described method and the following equation:

{[C(μg/mL) x35]/1000}/ weight of PLA MP, [Equation 4]

where C the concentration corresponded to the calibration curve and weight of PLA MP the precise amount of PLA microparticles weighted for the quantitative determination of model drugs.

Sample	Amount of Capreomycin(mg)	Average	±SD
1	0.0471	**0.0413**	**0.0051**
2	0.0374		
3	0.0394		

Table 5.2 Drug content of capreomycin in PLA microparticles

Sample	Amount of model drug B (mg)	Average	±SD
1	0.1299	**0.1174**	**0.0146**
2	0.1014		
3	0.1209		

Table 5.3 Drug content of model drug B in PLA microparticles

As has been already referred above, the drug content selected for the fabrication of PLA microparticles (theoretical drug content) was 20% w/w. The experimental drug content found was 4.13% concerning the model drug A-capreomycin and 11.74% regarding the model drug B. Thus, the encapsulation efficiency of model drug A was 46.93% and of model drug B was 58.70%.

5.3 Quantification of model drug A and model drug B in leucine microparticles

The amount of model drug A-capreomycin and model drug B encapsulated into the leucine microparticles was calculated according to the already described method and according to the following equation:

{[C(μg/mL) x35]/1000}/ weight of Leucine MP, [Equation 5]

where C the concentration corresponded to the calibration curve and weight of Leucine MP the precise amount of leucine microparticles weighted for the quantitative determination of model drugs.

Sample	Amount of Capreomycin(mg)	Average	±SD
1	0.0442	**0.0426**	**0.0032**
2	0.0447		
3	0.0389		

Table 5.4 Drug content of capreomycin in leucine microparticles

Sample	Amount of model drug B (mg)	Average	±SD
1	0.0742	**0.0693**	**0.0061**
2	0.0712		
3	0.0625		

Table 5.5 Drug content of model drug B in leucine microparticles

The drug content selected for the fabrication of leucine microparticles (theoretical drug content) was 20% w/w. The experimental drug content found was 4.26% concerning the model drug A-capreomycin and 6.93% regarding the model drug B. Thus, the encapsulation efficiency of model drug A was 48.41% and of model drug B was 34.65%)

5.4 Particle size analysis

Leucine microparticles obtained by nano spray-drying showed a mean particle size 1.85 μm and a narrow size distribution which can be explained by the selected production method, whereas PLA microparticles exhibited a larger mean particle size 4.49 μm and a broader distribution (Figure 5.1, Figure 5.2). The broad distribution of PLA microparticles could be

rendered to the cohesiveness that characterizes the polylactic polymers and thus the tendency for formation of agglomerates.

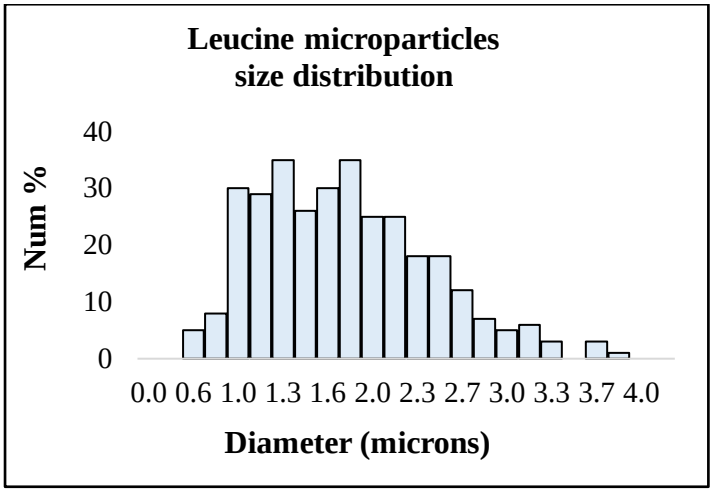


Figure 5.1 Leucine microparticles size distribution

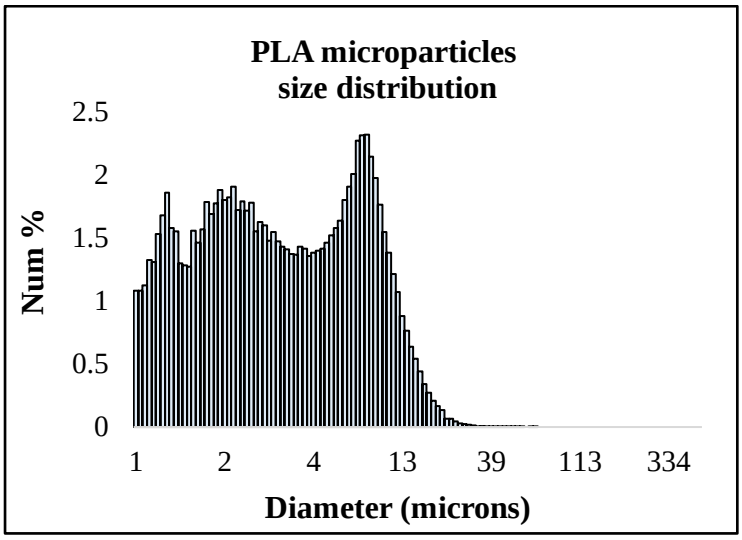


Figure 5.2 PLA microparticles size distribution

5.5 Tapped density and content uniformity analysis

Mixing conditions	%RSD (Content Uniformity)	Hausner ratio (Flowability)
30/70 PLA/Leucine blend ratio mixed for 15 minutes	2.80	1.29
50/50 PLA/Leucine blend ratio mixed for 10 minutes	4.01	1.25
70/30 PLA/Leucine blend ratio mixed for 15 minutes	5.67	1.34
30/70 PLA/Leucine blend ratio mixed for 5 minutes	4.19	1.25
70/30 PLA/Leucine blend ratio mixed for 5 minutes	4.19	1.73

Table 5.6 Summary of content uniformity and Hausner ratio values of blends

All blends appeared to be homogenous and %RSD was smaller than 6%. The most homogenous blend was the one with 30/70 PLA/Leucine blend ratio mixed for 15 minutes. Regarding the flowability properties of all blends, they can be characterized as passable except for the 70/30 PLA/Leucine blend ratio mixed for 5 minutes, of which the flowability is characterized as poor, according to the flowability scale described in the Eur. Pharmacopeia (Edition 9) (Table 5.6).

5.6 Aerodynamic assessment

The aerodynamic assessment of unblended PLA and leucine microparticles as well as their blends of the experimental design study, was carried out in means of Twin-stage impinger, according to the method described above. After each twin-stage procedure, two principal indicators were calculated in order to express the aerodynamic performance of PLA and leucine microparticles and their blends; the fine particle fraction (%FPF) and emitted dose (%ED) as follows:

%FPF= (mg of drug deposited onto stage 2)/ [(mg of drug deposited onto stage 1)+ (mg of drug deposited onto stage 2)]

[Equation 6]

%ED= [(mg of drug deposited onto stage 1)+ (mg of drug deposited onto stage 2)]/ mg of total drug deposition

[Equation 7]

5.6.1 Aerodynamic assessment of unblended PLA and leucine microparticles

Type of microparticles	%FPF (Fine Particle Fraction)	±SD	%ED (Emitted Dose)	±SD
Leucine MP	**77.13**	0.0151	**91.29**	0.0170
PLA MP	**11.81**	0.0162	**91.12**	0.0196

Table 5.7 Summary of %FPF and %ED values of unblended PLA and leucine microparticles

As expected, leucine microparticles exhibited excellent aerodynamic properties not only thanks to their small geometric size, but also due to the intrinsic anti-adherent properties that leucine presents (Moghaddam et al., 2013). The %Fine Particle Fraction (%FPF) of leucine microparticles was impressively high and more specifically reached to 77%. On the other hand, as had been supposed, the cohesive properties that characterize the poly-lactic polymers and consequently the PLA microparticles, constitute the main obstacle regarding their respirability performance. Hence, despite their small geometric size, they had an extremely poor TSI (Twin-Stage Impinger) deposition, lower than 12% (Table 5.7, Figure 5.3, Figure 5.4). Although both PLA and Leucine microparticles exhibited potentially suitable geometric size, their aerodynamic performance was completely different. As has been already referred above, the particle size is only one parameter that should be taken into account when evaluating the aerodynamic performance of inhalable particles. In fact, the irregular shape and surface that leucine microparticles exhibited (as shown in SEM images below), could positively influence the powder aerodynamic properties. According to the equation of the aerodynamic diameter ($d_A \cong d_G \sqrt{\rho/\chi\rho_0}$), already mentioned above, the irregular shape (high χ value) could decrease the particle aerodynamic diameter (since the dynamic shape factor is inversely proportional to

the d_A) and thus enhance the aerodynamic performance (Schoubben et al., 2013). However, the %Emitted Dose (%ED) was the same for both types of microparticles and equally high (91%). The data presented are in detail described in Appendix 1.

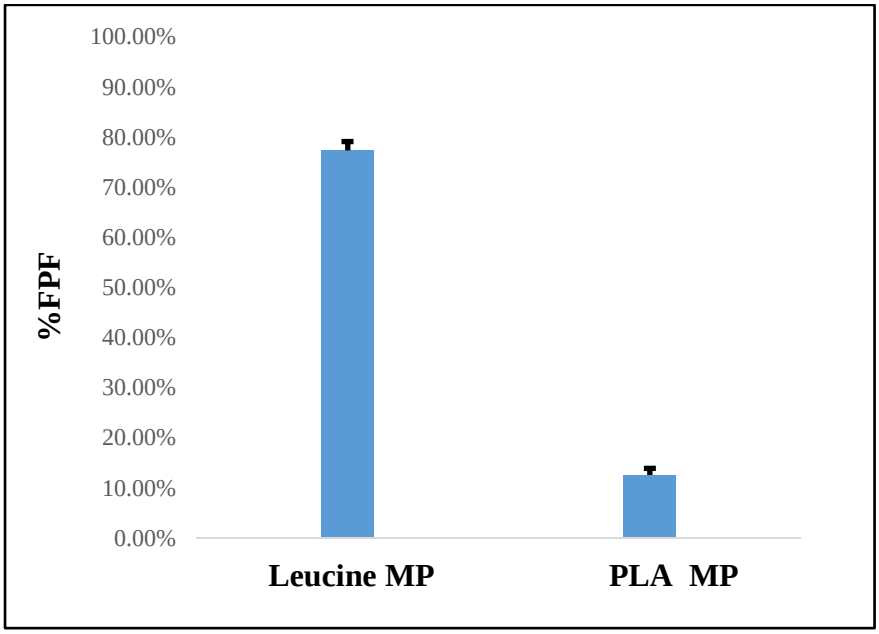


Figure 5.3 %Fine Particle Fraction (%FPF) of PLA and leucine microparticles.

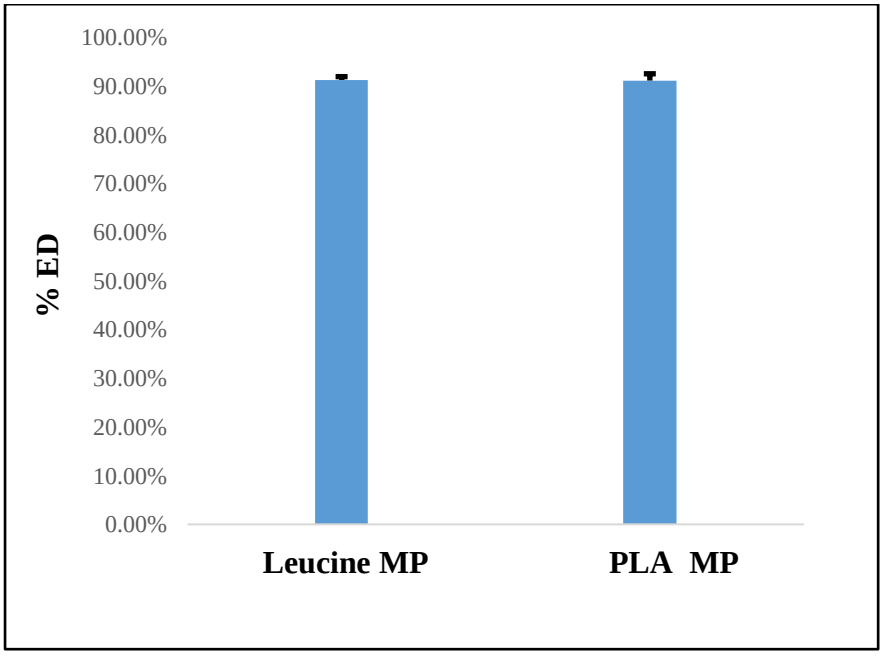


Figure 5.4 %Emitted dose (%ED) of PLA and leucine microparticles.

5.6.2 Aerodynamic assessment of blends

Mixing conditions	%Emitted Dose (%ED)	±SD
30/70 PLA/Leucine blend ratio mixed for 15 minutes	96.31	0.0153
50/50 PLA/Leucine blend ratio mixed for 10 minutes	96.41	0.0025
70/30 PLA/Leucine blend ratio mixed for 15 minutes	95.81	0.0048
30/70 PLA/Leucine blend ratio mixed for 5 minutes	95.17	0.0144
70/30 PLA/Leucine blend ratio mixed for 5 minutes	95.83	0.0099

Table 5.8 Summary %ED values of blends.

The evaluation of the aerodynamic performance of various blends resulted from the 2^2 factorial design is presented in Tables 5.8 and 5.9, whereas the data are in detail described in Appendix 2. As shown in Table 5.8, all blends resulted from the experimental design study exhibited high values of emitted dose from the device (>95%). Table 5.9 demonstrates the blends as well as the triplicate of center point (50/50 PLA/Leucine blend ratio mixed for 10 minutes). The response selected for the experimental design was the Fine Particle Fraction (%FPF). Observing the %FPF values of various blends, it is obvious that the introduction of leucine microparticles in the formulation highly improved the %FPF values of all blends, from 11.81% (%FPF that PLA microparticles exhibited) to 53.23% regarding the blend with the best mixing conditions, whereas the effect of leucine appears to be non-linear. In general, except for the blend with 70/30 PLA/leucine blend ratio mixed for 5 minutes, all mixtures showed %FPF higher than 46%, verifying the initial hypothesis concerning the improvement of aerosolization properties of PLA microparticles when mixed with Leucine microparticles (Sou et al., 2013). More specifically, the mixture with the best aerodynamic properties is the one with 30/70 PLA/Leucine blend ratio mixed for 15 minutes, which exhibits the highest %FPF value 53.66% (Table 5.9). On the other hand, the blend with 70/30 PLA/Leucine blend ratio mixed for 5

minutes seems to have the lowest %FPF value 34.70% (Table 5.9). Since leucine has been characterized as surface-active excipient, it may absorb at the PLA microparticle' surface reducing their contact area and the cohesiveness, by modifying the surface morphology (Eedara et al., 2018). This was verified after observing the SEM images of blends, which are presented below. Consequently, the improvement of aerosolization properties of PLA microparticles is undeniable when comparing the %FPF value of PLA microparticles (11.81%) to the %FPF value even of the blend with the lowest %FPF value (34.70%).

Run	**Factor A** **Mixing time (min)**	**Factor B** **PLA/leucine blend ratio (%w/w)**	**Response** **%FPF**
1	-1	-1	**46.88**
2	1	-1	**53.66**
3	-1	1	**34.70**
4	1	1	**49.64**
5	0	0	**48.19**
6	0	0	**52.07**
7	0	0	**53.23**

Table 5.9 %FPF values of blends (Design- Expert Version 10.0.7).

5.7 Experimental design results

In order to evaluate the influence of process parameters on %FPF value of blends, the Pareto chart is presented in Figure 5.5. The statistically significant factors are those which exhibit p-value <0.05 at a 95% confidence interval. The mixing time (Factor A) seems to be the most influential factor, as verified by the statistical analysis (Analysis of Variance, ANOVA) with p-value 0.0008, which is presented below (Table 5.10). The blends mixed for 15 minutes exhibited higher %FPF values than those mixed for 5 minutes. It can be hypothesized that as the mixing time increases, the blends become more homogeneous and as a result leucine microparticles are surrounded in a better way to the PLA microparticles' surface. On the other

hand, blends mixed for 5 minutes appear to have lower deposition onto stage 2 of TSI (Twin-Stage impinger) and thus lower %FPF values. This mixing time is not enough to produce homogenous blends resulting in the formation of large agglomerates of PLA microparticles, which are not able to pass to the lower TSI stage. Moreover, the PLA/Leucine blend ratio (Factor B) is also statistically important process parameter as can be verified by the ANOVA statistical analysis, exhibiting p-value 0.0058 (Figure 5.5, Table 5.10). As expected, the blends with higher PLA/Leucine blend ratio present lower %FPF values. Nevertheless, we should also take into consideration the interaction between the two parameters (AB interaction) which is not a statistically important model term (p-value 0.1135 since values greater than 0.1000 indicate the model terms are not significant); though it can provide further information. As shown in Figure 5.6, the effect of blend ratio on %FPF value is higher when factor A (mixing time) is at low levels, whereas this influence is smoothed out at high levels of factor B (PLA/Leucine blend ratio). This is the reason why the mixing time is a more influential factor than the PLA/Leucine blend ratio and at the same time verifies the non-linear effect of leucine on the aerodynamic performance of blends.

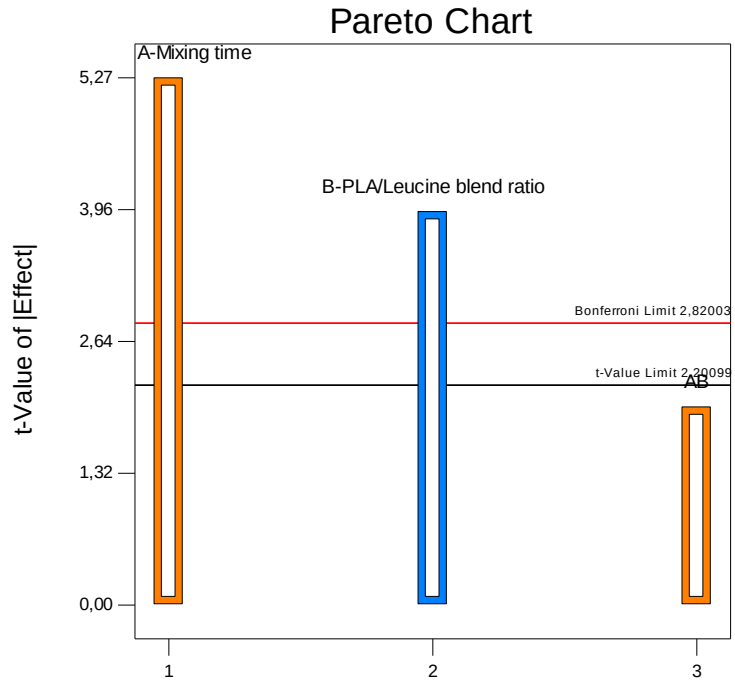


Figure 5.5 Pareto chart indicating the influential factors (Design- Expert Version 10.0.7).

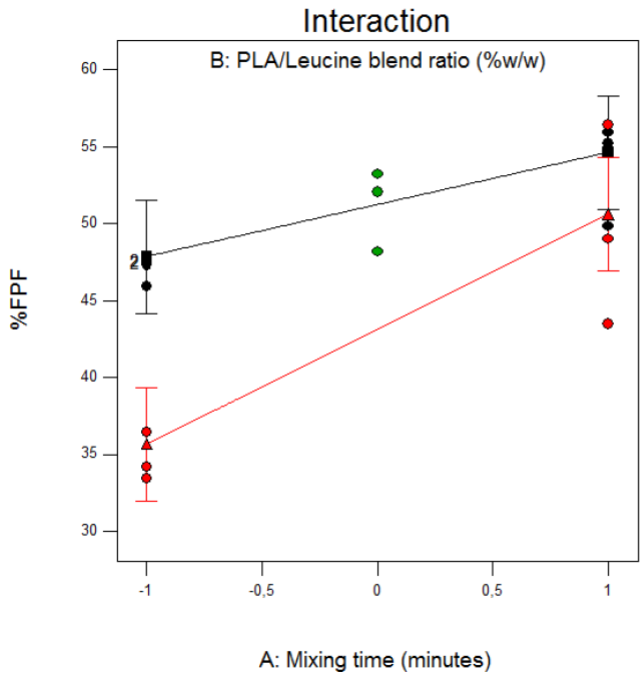


Figure 5.6 Interaction plot indicating the interaction between the two process parameters (Design-Expert Version 10.0.7).

Statistical Analysis: Analysis of Variance (ANOVA)

Source	Sum of squares	df	Mean square	F Value	p-value Prob>F	
Model	600.75	3	200.25	11.86	0.0009	significant
A-Mixing time	353.82	1	353.82	20.95	0.0008	
B-PLA/Leucine blend ratio	196.99	1	196.99	11.66	0.0058	
AB	49.94	1	49.94	2.96	0.1135	
Residual	185.79	11	16.89			
Lack of Fit	58.61	1	58.61	4.61	0.0574	not significant
Pure Error	127.18	10	12.72			
Cor Total	786.54	14				

Table 5.10 ANOVA (Analysis of Variance) for selected factorial model (Design- Expert Version 10.0.7.

As far as the model is concerned, the model F-value of 11.86 implies the model is significant and there is only a 0.09% chance that an F-value this large could occur due to noise. According to the ANOVA statistical analysis, the selected model is statistically significant with p-value 0.0009 (<0.05) at a 95% confidence interval. The Lack of Fit F-value of 4.61 implies there is a 5.74% chance that a Lack of Fit F-value this large could occur due to noise.

R-Squared	0.7638
Adjusted R-Squared	0.6994
Predicted R-Squared	0.5710
Adequate Precision	8.936

Table 5.11 Model statistical terms (Design- Expert Version 10.0.7).

- ✓ According to the correlation coefficient R^2, the 76.38% of the variability can be rendered to the selected model
- ✓ The difference between the adjusted R^2 and the predicted R^2 is smaller than 0.2 and consequently there is no need for model transformation
- ✓ The precision of the model is sufficient with value 8.936 higher than 4 (Table 5.11).

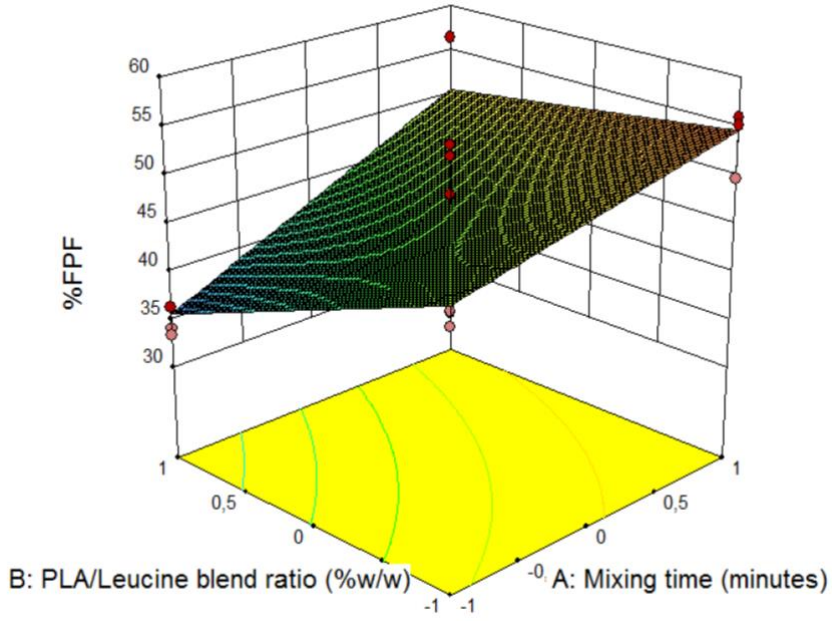


Figure 5.7 3D plot depicting the effects of the two factors on %FPF (Design- Expert Version 10.0.7).

Overall, as shown in Figure 5.7, the model suggests an improvement of blend aerodynamic behavior either increasing mixing time or decreasing PLA/leucine ratio.

5.8 Particle morphology

The morphology of PLA and leucine microparticles as well as their blends was evaluated through scanning electron microscopic (SEM) images. Spray dried PLA microparticles appear in general to be spherical with a relatively smooth and closed surface, as has been already reported before (Arpagaus and Schafroth, 2009) (Figure 5.8). On the other hand, leucine microparticles show an irregular morphology and porous surface (Figure 5.9).

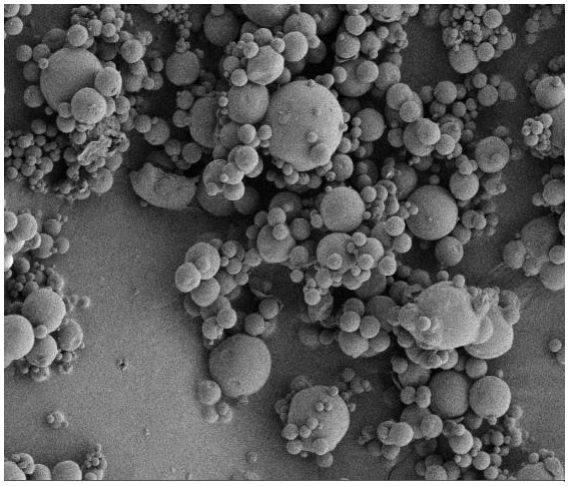


Figure 5.8 Spray-dried PLA microparticles.

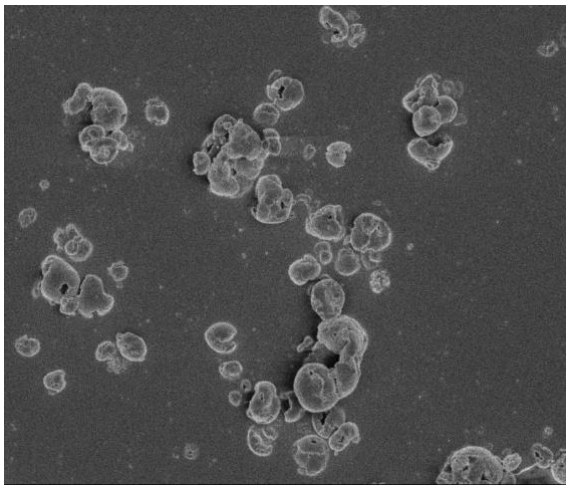


Figure 5.9 Spray-dried leucine microparticles.

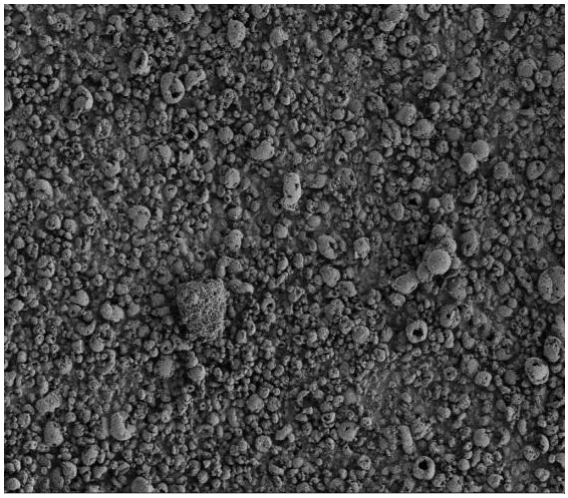


Figure 5.10 30/70 PLA/Leucine blend ratio mixed for 15 minutes.

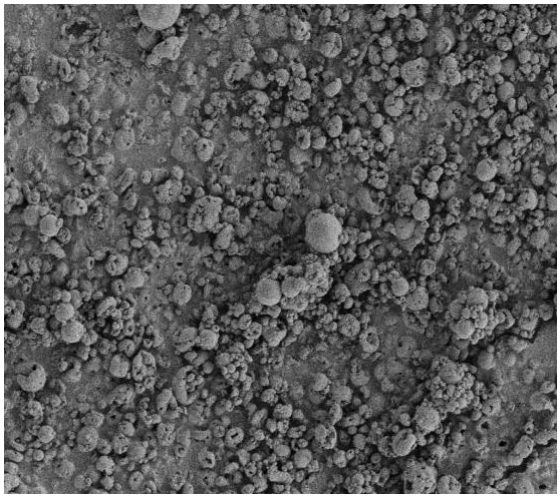


Figure 5.11 50/50 PLA/Leucine blend ratio mixed for 10 minutes

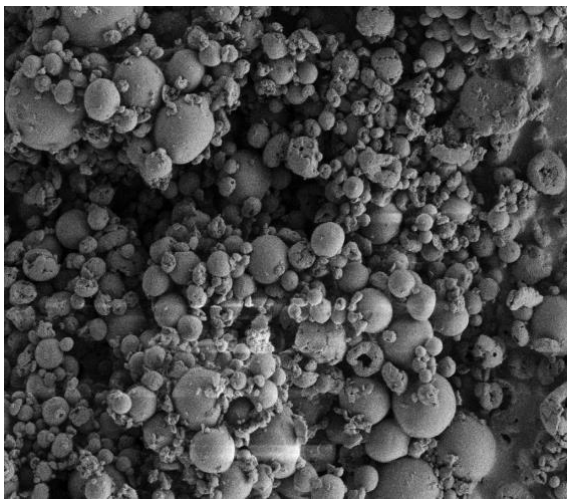


Figure 5.12 70/30 PLA/Leucine blend ratio mixed for 15 minutes.

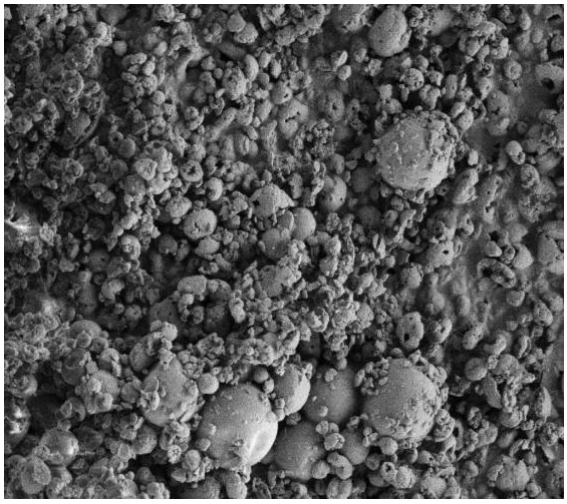


Figure 5.13 30/70 PLA/Leucine blend ratio mixed for 5 minutes

Observing the SEM images of blend with 30/70 PLA/Leucine microparticles mixed for 15 minutes (Figure 5.10), it can be noted that the Leucine microparticles with the irregular morphology are dominant, since they consist they 70% of the mixture. The absence of large agglomerates, the homogeneity of the mixture as well as the dispersibility enhancement through the incorporation of leucine could be possible explanations regarding the satisfying aerodynamic properties that this blend exhibited. In addition, the blend with 70/30 PLA/Leucine blend ratio mixed for 15 minutes (Figure 5.12) presents larger PLA microparticles surrounded by smaller microparticles of leucine, verifying the initial hypothesis concerning the glidant properties that the amino-acid exhibits.

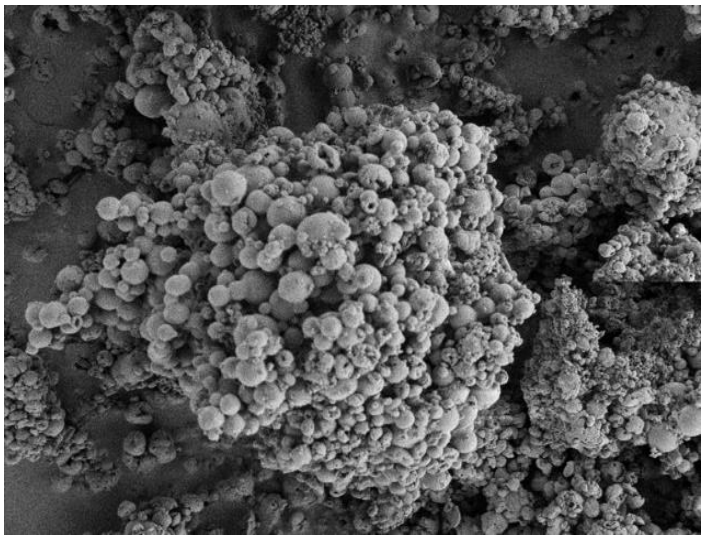
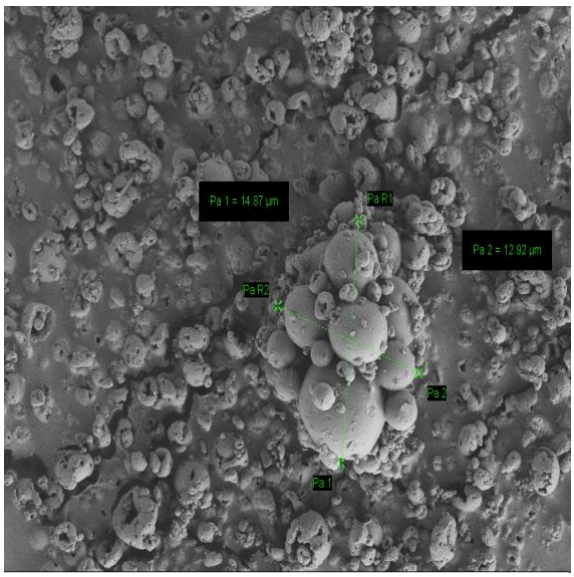


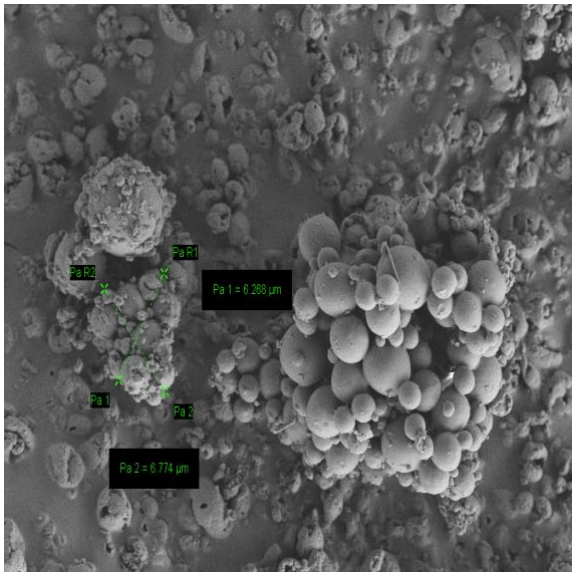
Figure 5.14. 70/30 PLA/Leucine blend ratio mixed for 5 minutes.

From the other side, the blend with 70/30 PLA/Leucine blend ratio mixed for 5 minutes exhibits large agglomerates of the PLA microparticles (Figure 5.14). In this blend, the strong cohesive forces between the PLA microparticles result in the formation of aggregates, whereas the short mixing time is not enough for the de-aggregation and the manifestation of leucine's effect. Consequently, it can be supposed that these aggregates are the reason why this blend had a poor TSI (Twin-Stage) deposition.

In order to understand thoroughly the impact of leucine and the aerosolization behavior of the mixture, the 30/70 PLA/Leucine blend mixed for 15 minutes was dispersed and the powder, after aerosolization, was collected from the TSI and was morphologically analyzed. As shown in Figure 5.15 (a, b), there are still some separated leucine microparticles, which are able to pass through the lower TSI stage (Stage 2) as well as PLA and leucine microparticles agglomerates.



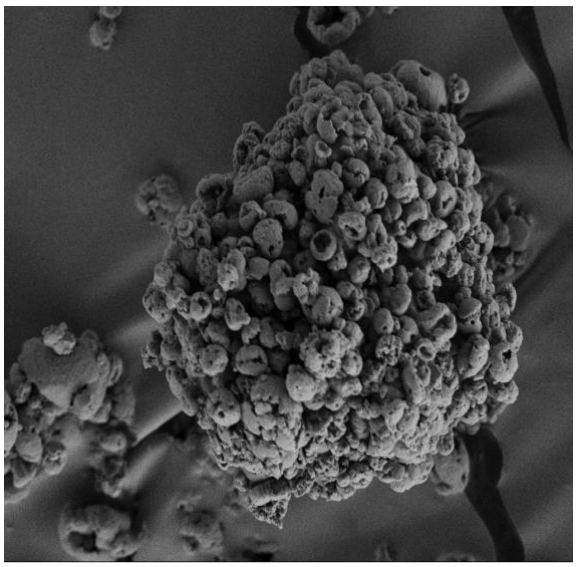
a



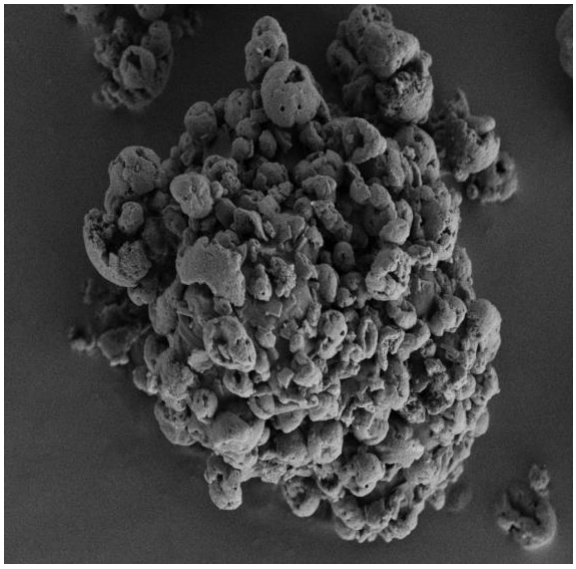
b

Figure 5.15 (a, b) 30/70 PLA/Leucine blend ratio mixed for 15 minutes after nebulization.

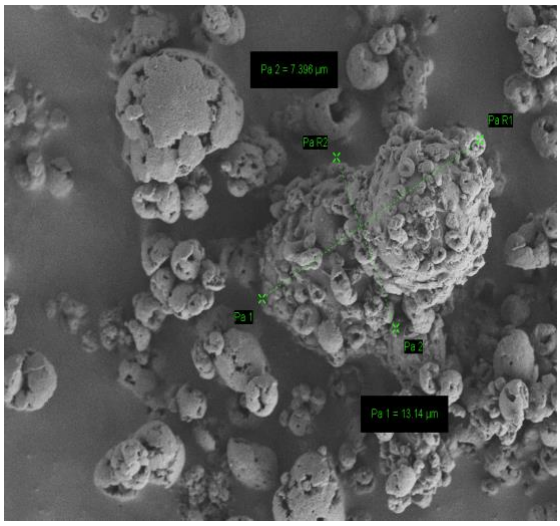
It seems that the aerodynamic behaviour of the mixture depends on the size of these agglomerates. In particular, agglomerates between smaller PLA microparticles surrounded by the glidant leucine microparticles with geometric size lower than 6.5 μm (Figure 5.15 b) may be able to deposit onto the lower TSI stage. However, aggregates between larger PLA microparticles, visible in Figure 5.15 a, may deposit onto the upper TSI stage (Stage 1).



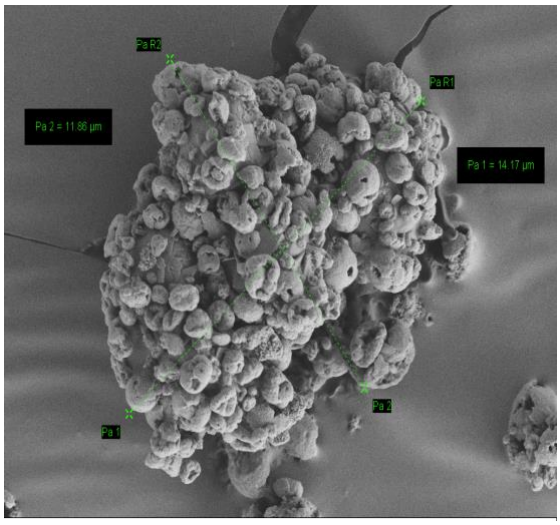
a



b



c



d

Figure 5.16 (a, b) 20/80 PLA/Leucine blend ratio mixed for 15 minutes,
(c, d) 20/80 PLA/Leucine blend ratio mixed for 15 minutes after nebulization.

As stated above, the obtained DoE model predicted further improvement of aerodynamic properties if either mixing time is increased or PLA/leucine blend ratio is decreased. In order to verify such a prediction, a blend with 20/80 PLA/Leucine blend ratio mixed for 15 minutes was prepared and aerodynamically and morphologically characterized. An unexpected drop in %FPF (36%) was measured. This result was explained by the large agglomerates displayed in Figure 5.16 (a, b) with a geometric diameter higher than 10 µm (measured manually through Image J), which are the likely result of leucine MP aggregation on PLA MP. These clustered

MP were difficult to disperse upon insufflation thus suggesting their likely role in the %FPF reduction measured [Figures 5.16 (c, d)].

5.9 Drug release testing

Drug release testing was performed according the method described above for PLA and leucine microparticles and the blend which exhibited the best aerodynamic performance (expressed as %FPF value), namely the 30/70 PLA/Leucine blend ratio mixed for 15 minutes. Model drug A, capreomycin sulfate, showed an expected dual release. The 70.94% of capreomycin was liberated from microparticles at the first 5 hours, whereas the rest 20% at 30 hours, where the 91.4% of encapsulated drug was found in the outer media (Figure 5.17). However, the model drug B showed a faster release and the 89.35% of drug was liberated at 5 hours (Figure 5.17). It has to be noted that due to the degradation observed concerning the model drug B after 24 hours in Phosphate Buffer 0.01 M; pH 7.4, used as release media during the drug release testing, the values were corrected according to the stability test performed under the same conditions (Figure 5.18).

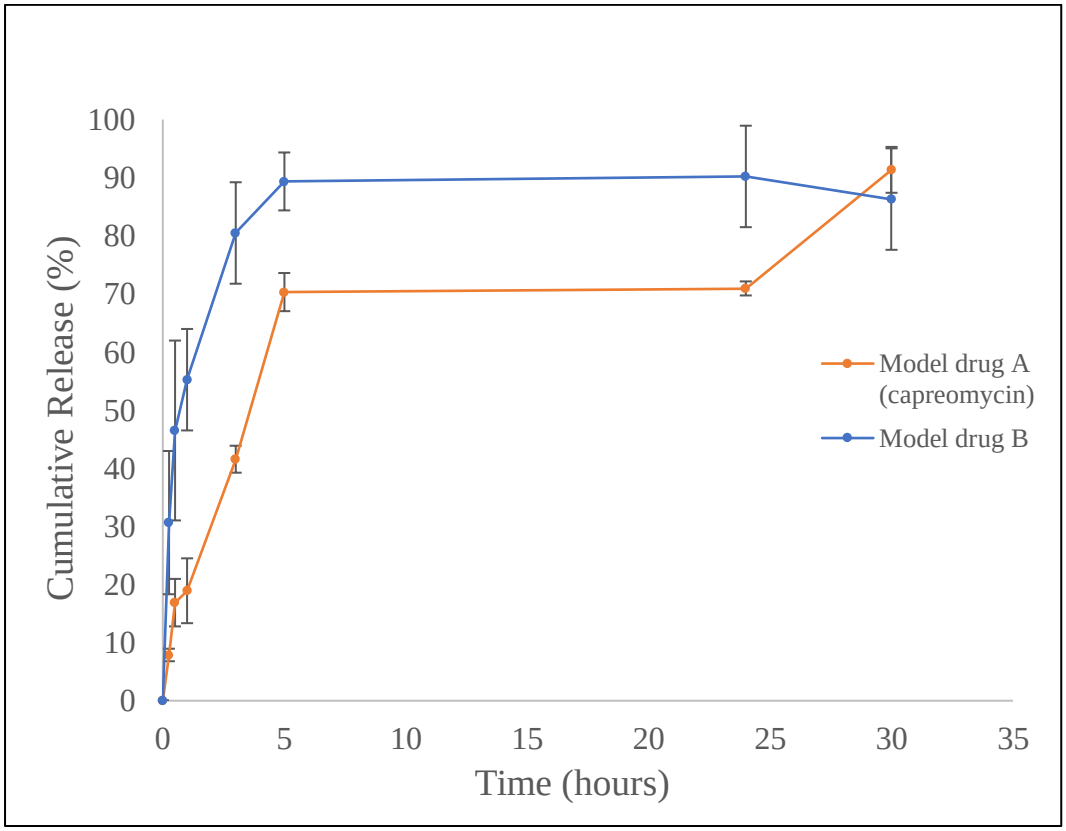


Figure 5.17 Cumulative release of capreomycin and model drug B from blend with 30/70 PLA/Leucine ratio mixed for 15 minutes.

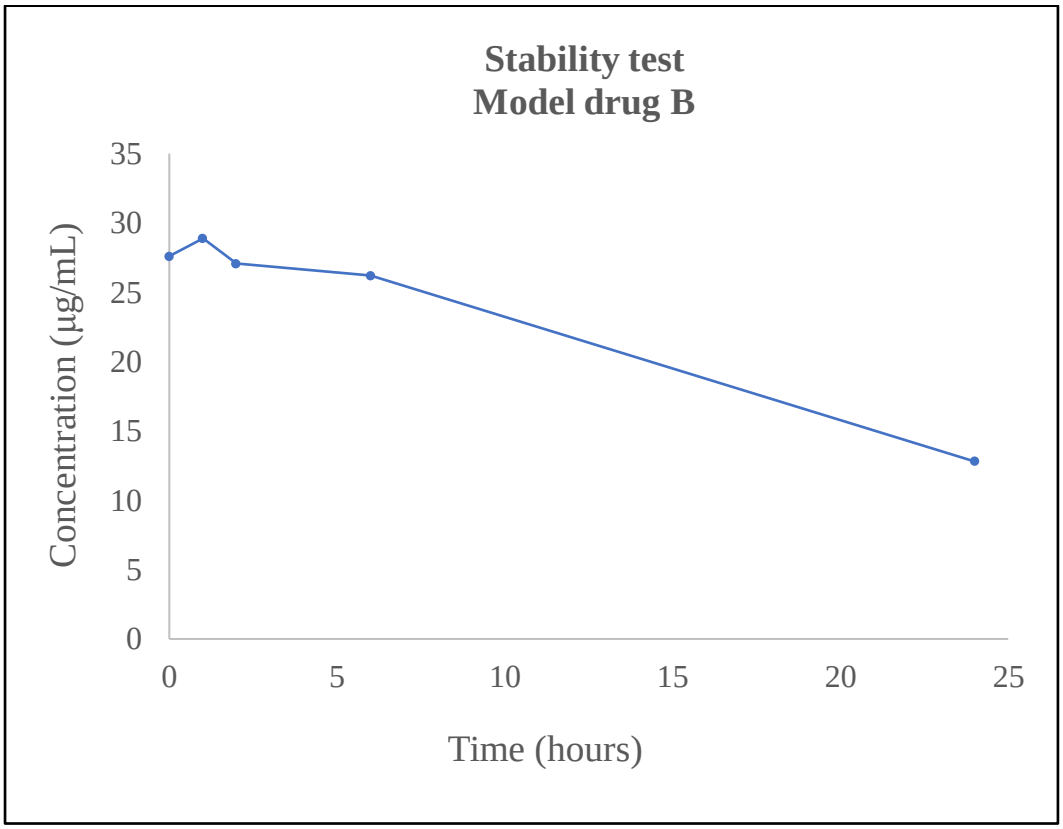


Figure 5.18 Model drug B stability test.

With the aim to evaluate the effect of leucine and PLA on the drug release from microparticles, in Figure 5.19 are presented the release profiles of model drug B from leucine and PLA microparticles. The 85.54% of model drug B was liberated from leucine microparticles within 3 hours, whereas only the 23.94% of model drug B was released from PLA microparticles at the same time point and the total release lasted 48 hours (Figure 5.19). The faster release from leucine microparticles can be rendered to the high water-solubility that leucine exhibits, resulting in the burst release of drug (Figure 5.19). Hence, in this case the drug release profile depends on the solubility of drug in the inner media and its diffusion through the dialysis membrane.

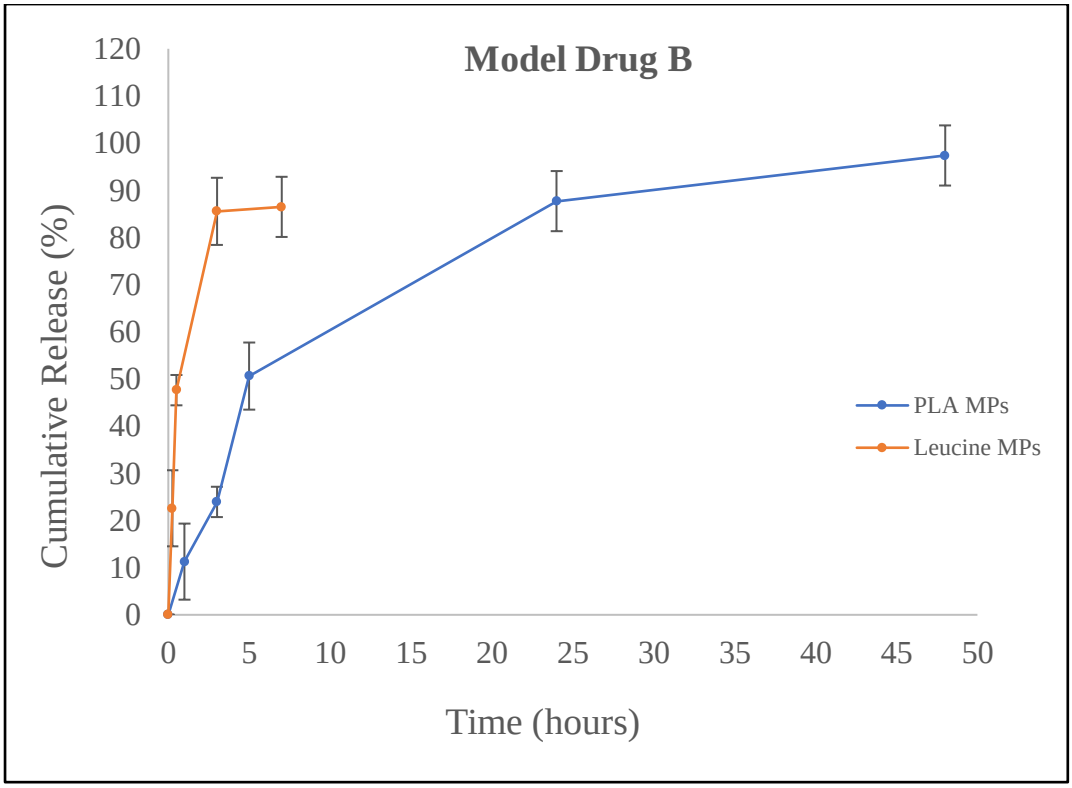


Figure 5.19 Cumulative release of model drug B from PLA and leucine microparticles.

From the other side, as a result of PLA biodegradation, a biphasic diagram was observed (Figure 5.19). The initial release was detected during the first 5 hours (approximately 50% of loaded drug). This burst release could be related to the fraction of drug on the surface of the MPs. The rate of drug release decreased after the first 5 hours and the total drug release lasted 48 hours. This longer release of drug probably happens due to the progressive polymer degradation into oligomeric and monomeric products, creating a passage for the drug to be released until the complete polymer solubilization (Moghaddam et al., 2013).

5.10 Differential scanning calorimetry (DSC) analysis

Differential scanning calorimetry thermograms, displayed in Figure 5.20, revealed the absence of the typical sharp endothermic peak of model drug B, which reflects its crystalline form, in all the formulations as well as their blends. This phenomenon could occur due to either the absence of crystalline drug at least at the particle surface level and its molecular dispersion into the MP matrix or the amorphization of model drug B during the spray-drying process (Musumeci et al., 2006). Spray drying has been applied to produce amorphous pharmaceutical products since the fast drying and cooling regimen prevents crystal growth (Shen et al., 2010; Mu and Feng, 2001).

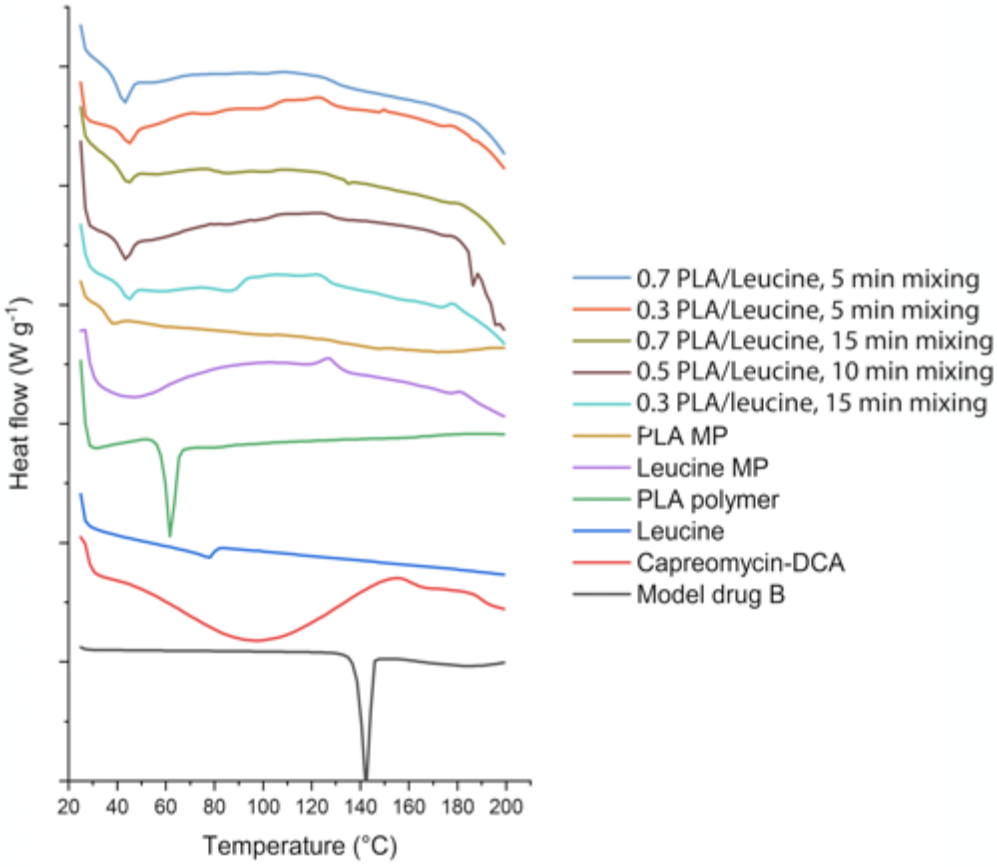


Figure 5.20 DSC diagram of capreomycin-DCA complex, PLA, leucine, PLA MPs, Leucine MPs as well as the mixture with the best blending conditions.

The advantage of amorphization is due to a higher Gibbs free energy and thus higher apparent solubility and faster dissolution rates of the solid, compared to thermodynamically stable crystalline forms (Einfalt et al., 2013; Omar et al., 2015; Pokharkar et al., 2006).

Since the polymer employed was an amorphous material, all blends and PLA profiles exhibited the typical step profile corresponding to the glass transition (Tg) between a stiffer and hindered

state of polymer chains to a rubbery and more relaxed condition. The initial Tg value that for PLA was around 58°C, dropped consistently in PLA MP and all blends to below 40°C. This shift is the consequence of drug loading as well as small amount of residual water (Jadhav et al., 2009). Overall, such an effect and the absence of additional signals in the profiles suggest that drugs were either homogeneously dispersed in the matrix or in an amorphous state. Similar considerations can be drawn for leucine MP that did not show evident transitions either as a raw material or drug loaded MP.

6. CONCLUSION

The initial hypothesis concerning the improvement of the aerosolization performance of PLA microparticles by incorporating into the formulation glidant leucine microparticles was verified. The double role of leucine particles was both the encapsulation of model drug A capreomycin sulfate and model drug B, avoiding the drug dilution, and the amelioration of aerodynamic properties of polymeric particles. Despite their small geometric size, PLA microparticles exhibited extremely low TSI (Twin-Stage) and %FPF value lower than 12%, whereas the idea of mixing with glidant leucine particles was successful, since all blends showed %FPF value higher than 34%. Increasing leucine particle fraction in the blend enhanced the %FPF up to nearly 50%. As expected, the best behaviour was obtained at 30/70 PLA/Leucine blend ratio. However, the leucine effect was not straightforward, suggesting that the improvement of aerodynamic properties is not linked only to the presence of a higher leucine MP fraction but to a real association between PLA and leucine MP. In fact, contrary to what suggested by the DoE model, increasing further leucine MP to 80% resulted in a poorer aerodynamic behaviour. This finding was explained by the tendency to cluster of leucine MP with PLA MP leading to not dispersible aggregates after insufflation.

Furthermore, the blend with the best blending conditions (30/70 PLA/Leucine blend ratio mixed for 15 minutes) exhibited a dual drug release pattern, which could be favourable regarding the inhaled antibiotic therapy. Content uniformity in the blends was granted by the entrapment of both drugs in both formulations and this approach also prevented drug dilution that usually occurs when using conventional inhalation carriers, such as lactose or mannitol.

Nevertheless, despite the fact that all blends showed high emitted dose from the device values, none of the them approximated to the excellent aerodynamic properties that leucine particles had (%FPF> 77%). Future perspectives could include the thorough understanding of the aforementioned phenomenon and the proposal of a potential strategy, such as increasing the leucine MP particle size, for obtaining the above proposed formulation with even better aerosolization properties.

APPENDIX 1

- *Leucine microparticles*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0143	**1,1568**	**78.37%**	**90.75%**
Device	0.0927			
Stage 1	0.2271			
Stage 2	0.8227			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0197	**1,2042**	**78.37%**	**91.14%**
Device	0.0870			
Stage 1	0.2374			
Stage 2	0.8601			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0415	**1,2900**	**75.32%**	**91.98%**
Device	0.0619			
Stage 1	0.2929			
Stage 2	0.8937			

4	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0162	**1,2472**	**76.46%**	**94.52%**
Device	0.0521			
Stage 1	0.2775			
Stage 2	0.9014			

- *PLA microparticles*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0352	**2.4517**	**11.63%**	**92.10%**
Device	0.1585			
Stage 1	1.9954			
Stage 2	0.2626			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0422	**2.2658**	**13.51%**	**90.14%**
Device	0.1812			
Stage 1	1.7666			
Stage 2	0.2759			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0347	**2.4027**	**10.28%**	**94.06%**
Device	0.1081			
Stage 1	2.0276			
Stage 2	0.2323			

APPENDIX 2

- *0.3 PLA/Leucine microparticles blend ratio mixed for 15 minutes*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0083	**1.6667**	**55.21%**	**97.67%**
Device	0.0306			
Stage 1	0.7290			
Stage 2	0.8988			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0116	**1.6116**	**55.95%**	**94.12%**
Device	0.0831			
Stage 1	0.6682			
Stage 2	0.8486			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0056	**1.1002**	**49.83%**	**91.98%**
Device	0.0826			
Stage 1	0.5077			
Stage 2	0.5043			

- *0.7 PLA/Leucine microparticles blend ratio mixed for 15 minutes*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0056	**1.8967**	**56.44%**	**95.69%**
Device	0.0762			
Stage 1	0.7905			
Stage 2	1.0244			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0119	**1.8708**	**49.00%**	**95.41%**
Device	0.0740			
Stage 1	0.9103			
Stage 2	0.8747			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0061	**1.6867**	**43.48%**	**93.13%**
Device	0.1098			
Stage 1	0.8878			
Stage 2	0.6830			

- *0.3 PLA/Leucine microparticles blend ratio mixed for 5 minutes*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0085	**1.3533**	**47.42%**	**96.77%**
Device	0.0351			
Stage 1	0.6886			
Stage 2	0.6210			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0219	**1.2083**	**47.31%**	**95.64%**
Device	0.0307			
Stage 1	0.6089			
Stage 2	0.5468			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0284	**1.4354**	**45.92%**	**94.00%**
Device	0.0577			
Stage 1	0.7297			
Stage 2	0.6195			

- *0.7 PLA/Leucine microparticles blend ratio mixed for 5 minutes*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0251	**1.7063**	**36.46%**	**94.78%**
Device	0.0639			
Stage 1	1.0276			
Stage 2	0.5897			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0185	**1.7417**	**33.44%**	**96.74%**
Device	0.0382			
Stage 1	1.1215			
Stage 2	0.5634			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0053	**1.6160**	**34.42%**	**93.29%**
Device	0.1031			
Stage 1	0.9887			
Stage 2	0.5189			

- *0.5 PLA/Leucine microparticles blend ratio mixed for 10 minutes (Run 3)*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0127	**2.0605**	**44.97%**	**96.95%**
Device	0.0502			
Stage 1	1.0993			
Stage 2	0.8983			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.1303	**2.0355**	**44.63%**	**90.21%**
Device	0.0690			
Stage 1	1.0167			
Stage 2	0.8195			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0888	**1.7952**	**54.98%**	**86.75%**
Device	0.1524			
Stage 1	0.6996			
Stage 2	0.8544			

- *0.5 PLA/Leucine microparticles blend ratio mixed for 10 minutes (Run 10)*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0087	**1.4970**	**57.12%**	**96.04%**
Device	0.0506			
Stage 1	0.6165			
Stage 2	0.8213			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0115	**1.4369**	**56.76%**	**94.84%**
Device	0.0627			
Stage 1	0.5893			
Stage 2	0.7735			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0046	**1.4316**	**45.82%**	**98.38%**
Device	0.0186			
Stage 1	0.7631			
Stage 2	0.6453			

- *0.5 PLA/Leucine microparticles blend ratio mixed for 10 minutes (Run 12)*

1	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0094	**1.7815**	**57.98%**	**96.14%**
Device	0.0594			
Stage 1	0.7197			
Stage 2	0.9930			

2	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0081	**1.7086**	**53.56%**	**96.46%**
Device	0.0524			
Stage 1	0.7654			
Stage 2	0.8827			

3	mg	SUM	%FPF (Fine Particle Fraction)	%ED (Emitted Dose)
Capsule	0.0093	**1.6502**	**44.66%**	**95.90%**
Device	0.0583			
Stage 1	0.8757			
Stage 2	0.7069			

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