University of Szeged

Faculty of Pharmacy

Institute of Pharmaceutical Technology and Regulatory Affairs

Head: *Dr. habil. Ildikó Csóka PhD*

PhD thesis

DEVELOPMENT OF DRY POWDER INHALATION SYSTEMS USING CIPROFLOXACIN HYDROCHLORIDE: NEW ASPECTS OF FORMULATION BY MEANS OF QUALITY BY DESIGN APPROACH

By:

Keyhaneh Karimi

Doctor of Pharmacy

Supervisors:

Dr. Habil Ildikó Csóka PhD

and

Dr. Habil Rita Ambrus PhD

SZEGED

2019

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

1. Pallagi E., **Karimi K**., Ambrus R.., Szabó-Révész P., Csoka I.
   New aspects of developing a dry powder inhalation formulation applying the quality-by-design approach
   International Journal of Pharmaceutics 511 (2016) 151-160
   IF.: 3.649 (Q1)
2. **Karimi K.**, Pallagi E., Szabó-Révész P., Csoka I., Ambrus R.
   Development of a microparticle-based dry powder inhalation formulation of ciprofloxacin hydrochloride applying the quality by design approach
   Drug Design, Development and Therapy Journal 10 (2016) 3331-3343
   IF.: 2.822 (Q1)
3. **Karimi K.,** Katona G., Csoka I., Ambrus R.
   Physicochemical stability and aerosolization performance of dry powder inhalation system containing ciprofloxacin hydrochloride
   Journal of Pharmaceutical and Biomedical Analysis 148 (2018) 73-79
   IF.: 2.983 (Q1)
4. Csoka I., **Karimi K.**, Mukhtar M., Ambrus R.
   Pulmonary drug delivery: Role of antibiotic formulations for treatment of respiratory tract infections
   *Acta Pharm Hung (*2019) *in press*
   IF: - (Q4)

PRESENTATIONS

Verbal presentation

Karimi K., Szabó-Révész P., Ambrus R. *Preparation of microcomposite-based drug delivery system for DPI formulation of ciprofloxacin. 3rd* Conference on Bio-based polymers and composites, Szeged, Hungary 2016.

Poster presentation

Karimi K., Ambrus R., Csoka I. *Development of antibiotic dry powder inhalation system based on Quality by Design methodology.* 12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs, Szeged, Hungary 2018.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude for the invaluable advice and kindhearted support of Dr. Ildikó Csóka, without whom this work would not have been possible. Her guidance helped me in all the time of research and writing of this thesis.

Special thanks to Dr. Rita Ambrus for her great help and patience, motivation, and immense knowledge throughout this work.

Besides my advisor, I would like to thank Prof. Piroska Szabó-Révész for her insightful comments and encouragement, which incented me to widen my research from various perspectives.

I also would like to recognize the amicable assistance of the 4th team of Institute of Pharmaceutical Technology and Regulatory Affairs as well as the whole Institute.

Last but not the least, I would like to thank my family: my parents and to my brother for supporting me spiritually throughout writing this thesis and PhD period.

CONTENT

ABBREVIATIONS

ACI – Andersen Cascade Impactor

CD – Hydroxypropyl beta Cyclodextrin

CIP – Ciprofloxacin Hydrochloride

CF – Cystic Fibrosis

CQA – Critical Quality Attributes

CPP – Critical Process Parameters

DPI – Dry Powder Inhalation

DSC − Differential Scanning Calorimetry

EMA – European Medicines Agency

ED – Emitted Dose

FDA – U.S. Food and Drug Administration

FPF – Fine Particle Fraction

FT-IR – Fourier Transform Infrared Spectroscopy

ICH – International Council Harmonization

LEU – L-leucine

MMAD – Mass Median Aerodynamic Diameter

PVA – Polyvinyl Alcohol3-88

QbD – Quality by Design

QTPP – Quality Target Product Profile

RH – Relative Humidity

RA – Risk Assessment

SEM – Scanning Electron Microscopy

SPD – Spray-Dried

TPP – Target Product Profile

TEER – The Transepithelial Electrical Resistance

XRPD – X-ray Powder Diffraction

1. INTRODUCTION

1.1 Pulmonary drug delivery and treatment of respiratory tract infection

Inhaled therapy for medicinal purposes was used at least 4,000 years ago but using antibiotics in pulmonary dosage form takes back to 1948 when Abbot Laboratories developed the aero-inhaler for inhalation penicillin G powder *(Anderson et al., 2005; Sanders, 2007)*. However large-scale therapeutic advance dates back in 1997, when tobramycin for inhalation was approved by the U.S. Food and Drug Administration (FDA) for use in patients with cystic fibrosis(CF) (*Konstan et al., 2011*).

Respiratory tract infections affect people in all ages and are very common *(Adi et al., 2010; Antoniu and Cojocaru, 2012)*. Globally, infections of the lower respiratory tract are among the top three major causes of morbidity and every year, they can be responsible for approximately 3.5 million deaths in the world *(Andrade et al., 2013; World Health Organization 2008)*. The most common treatment for respiratory infection involves oral or parenteral administration of high doses of single or combined antibiotics which can show undesirable side effect because of high systemic bioavailability *(Pilcer et al., 2013; Hoiby et al., 2011)*. The ability to deliver therapeutic agents to the site of action may allow efficient treatments of infection diseases to the respiratory tract and has many advantages over other routes (*Gelperina et al., 2005*). The excessive surface area of the lungs which contains the sufficient capillary vessels lead to a rapid absorption and the absorbed drug can directly reach to the blood circulation therefore bypass the first- pass metabolism in the liver and can be targeted by non-invasive methods *(Sung et al., 2007; Wu et al., 2014)*. Therefore, the delivery of even low concentrations of antibiotics to the lung, the site of infection leads to much higher concentrations of antibiotics in the lung, while reducing systemic exposure and the risk of toxicity and yields therapeutic effects with smaller drug doses than the oral or parenteral route *(Yang et al., 2009; Cipolla et al., 2013)*. The other big advantage of using pulmonary dosage form of antibiotics in treatment of chronic infections is, that is not associated with pain and this should increase patient comfort and compliance, causing promoted treatment outcome, enhance the quality of life, shorten the hospitalization period and significantly decrease morbidity and mortality *(Littlewood et al., 2012; Greally et al., 2012).*

Ciprofloxacin is a fluoroquinolone antibiotic, a broad-spectrum synthetic agent. Its main mechanism of action is the inhibition of the bacterial enzymes DNA gyrase (topoisomerase II)

and topoisomerase IV, thus preventing bacterial DNA from uncoiling and duplicating, leading to cell death *(Masadeh et al., 2015; LeBel et al., 1988)*. It has potent and effective activity against a wide range of Gram-positive bacteria like *Staphylococcus* and *Bacillus* species and against most Gram-negative microorganisms like *Pseudomonas* species, and it is often used in the treatment of inhalation anthrax and other lung infections *(Zhao et al., 2009; Bolon et al., 2011)*. Liposomal ciprofloxacin for inhalation is presently in clinical trials for the treatment of respiratory diseases. Dry powder formulations of ciprofloxacin is in the advanced development stage (*Wilson et al., 2013*).

1.2 Marketed Products

In considering all of these advantages development of inhaled antibiotics to treat lung infection is a largely active field, with five approved products in the USA and further in the late stages of clinical progress (*Cipolla et al., 2013)*. However literature background show most of researches and investigations of pulmonary dosage form of antibiotics focusing on treatment of CF than generally the treatment of respiratory tract infection *(O'Sullivan et al., 2009; Davis, 2006)*. CF is an inherited disease caused by different mutations of the transmembrane conductance regulator gene, and consequently respiratory failure as follow through of adhesive mucus of lungs and chronic inflammation of respiratory tract (*Ng et al., 2014)*. Currently, CF is the particular pulmonary infection disease in which inhaled antibiotics have received FDA and European Medicines Agency (EMA) approval (*Quon et al., 2014)*. **Table I**. illustrates the products of antibiotics in pulmonary dosage form that are present on the market.

Table Ⅰ: *Marketed Products in pulmonary dosage form of antibiotics*

Name of Products	Name of antibiotic	Type of pulmonary dosage form	Year	Producer	Approval Agency
CAYSTON®	Aztreonam	Nebulization	2010	Gilead Science	FDA
ARIKAYCE®	Amikacin	Nebulization	2018	PARI pharma	FDA
TOBI PODHALER	Tobramycin	Dry Powder Inhalation	2013	Novartis	FDA
TOBI NOVARTIS	Tobramycin	Nebulization	2013	Novartis	FDA
COLONYCIN®	Colistimethate Sodium	Dry Powder Inhalation	1982	Teva	FDA
COLOBREATHE	Colistimethate Sodium	Dry Powder Inhalation	2012	Teva	EMA

1.3 Formulation aspects of Dry Powder Inhaler systems

Concerning the possible dosage forms for pulmonal delivery of antibiotics one can use a wide variety of formulations such as dry powder inhalation (DPI). The most important approach of DPI is that the time required for delivering each dose is short and even less than one-third the time needed for delivering same dose for nebulization and adherence of patients increase significantly. This fact is expected to improve patients' adherence *(Geller et al., 2007; Westerman et al., 2007).*

DPI formulations have been used for patient treatment more than 60 years but during this period, the fundamental formation of DPI have not significantly changed *(Weers et al., 2015; Islam et al., 2008)*. DPI have become the first choice of inhaled formulation in European countries *(Hamishehkar et al., 2012).* DPI of antibiotics are more stable, quicker administration and have less risk of microbial contamination than parallel liquid formulations *Sousa and Pereira, 2014; Blau et al., 2007).*

DPI are formulated micronized drug particles with aerodynamic particle sizes of less than 5µm (*Islam et al., 2008*). The entrance of DPI through the airways and the lung faces with three mechanisms for deposition of particles (*Heyder et al., 1986*). These three mechanisms are: impaction, gravitational sedimentation and diffusion (*Carvalho et al., 2011*).

- Inertial impaction is defined as inertial particle deposition on a surface airway. It appears near bifurcations of the airways driving to the large airways, where there is a large flow rate and accelerated changes in the direction of the airflow (*Grgic et al., 2004*). This mechanism is essential for large particles having a diameter bigger than 5 µm and is frequent in the upper airways like mouth, pharynx and large airways. The expectation of impaction is corresponding to **Eq. 1** where ø is the change in direction of the air ways, r is the airways radius, V is the airstream velocity, and V_r is the terminal settling velocity.

$$\frac{V_r\, V \sin \emptyset}{\mathrm{gr}} \tag{1}$$

- Gravitational sedimentation is determined by its size, density and residence time in the airways. This mechanism is important for particles greater than 0.5 µm to 3 µm, in the small airways *(Newman, 1985*). According to stokes law particle settling under gravity, will attain a constant terminal settling velocity (**Eq. 2.)** where ρ is the particle density g is gravitational constant, d is the particle diameter and ŋ is the air velocity.

$$V_t \frac{\rho g\, d^2}{18\eta} \quad (2)$$

- Brownian diffusion is produced by concussion and bombardment of small particles with molecules in the respiratory tract where the airflow is very low. This mechanism is very important for particles smaller than 0.5 µm. Diffusion is inversely is proportional to particle size. As stated in Stokes-Einstein equation (**Eq. 3.**) where D is the diffusion coefficient, k_b is Boltzmann's constant, T is the absolute temperature and d is the particle diameter and η is viscosity. *(Aulton, 2018; Batchelor et al., 1976).*

$$D = \frac{T k b}{3\pi \eta d} \quad (3)$$

Different deposition mechanisms are very important for particles with different size *(Hofmann et al., 2011*). Particles larger than 5 µm will deposit in the upper airways by inertial impaction mechanism. Those larger than 1 µm and smaller than 5 µm will deposit by gravitational sedimentation in the lower airways and particles smaller than 1 µm will deposit by Brownian diffusion in the stagnant air of the lower airways by Brownian diffusion. Particles smaller than 0.5 µm are too large for Brownian diffusion and too small for impaction or sedimentation and finally they were exhaled ineffectively (*Heyder et al., 1986).* Hence for adequate deposition in reach to central and alveolar parts of lung the optimal size of particles is in the region of 1-5 µm.

Conventionally DPI has been used as a formulation of micronized drug incorporated in carrier excipient (*Healy et al., 2014*). These carrier excipients such as lactose, mannitol, trehalose and so on ... had been applied for prevention of agglutination of particles because these small particles (1-5µm) due to high surface free energy tend to stick together. without the presence of carrier excipient the surface energy is reduced and the adhesive as well as cohesive forces are overcome, nevertheless, the flowability of API particles are limited. (*Hickey, 2003*). Moreover, DPI of antibiotics usually have large therapeutic doses (e.g. between 10 mg and 100 mg of antibiotics) thus the carrier causes difficulty in application of DPI due to the increased powder volume and the scaling down of the use of antibiotics via pulmonary dosage form (*Pilcer et al., 2013*). Around the last two decades there has been assumed a significant research on the design of carrier free system for DPI (*Healy et al., 2014*). Applying carrier free system enable the delivery of high dose antibiotics to the lungs possible by limiting the amount of excipient (*Yu*

et al., 2016). Carrier free formulations can be handled by coated particles by lipids, amino acids, polymer and so on or can be applied by mechano-fusion dry coating process *(Boraey et al., 2013; Raula et al., 2010; Pilcer et al., 2006).* **Fig. 1** shows the possible mechanisms of deposition in the respiratory system by different DPI systems.

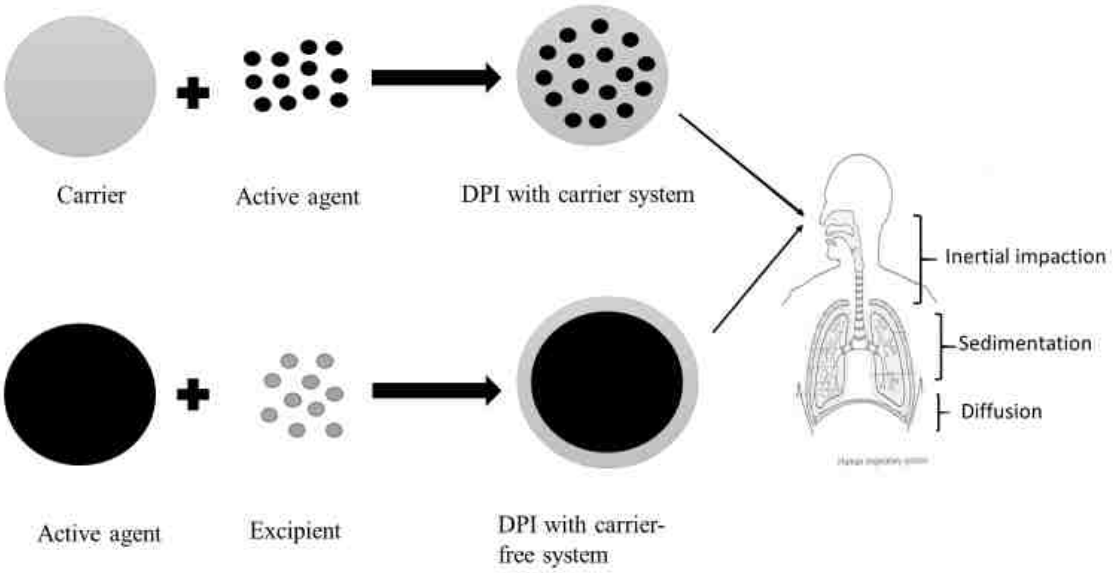


Figure 1: *Outline of different DPI systems with three particle deposition mechanisms taking place within the respiratory tract*

1.4 Quality by Design methodology

Quality by Design (QbD) is a holistic, systematic, risk, science and knowledge based method, focusing on extensive preliminary design in order to ensure the quality of medicinal products (*Yu et al., 2008*). The QbD method realizes a modern quality management thinking where the different quality requirements define the process's steps. In this case, risk assessment (RA) is considered to be the most accentual part, and the final goal is to ensure the predefined product quality. The QbD method can be applied to the entire pharmaceutical production process, or to certain parts of it but also in the early research and development phase *(Tomba et al., 2013; Huang et al., 2009; Zidan et al., 2007; Charoo et al., 2012).* Its use in the early developments can help in having a time and cost-effective process, in closing the gap between the science and the industry, as well as can facilitate the innovation transfer process to introduce new drugs to market *(Pallagi et al., 2015; Chatterjee et al., 2011).*

According to the current Regulatory Science philosophy, QbD has to be one of the key elements of different pharmaceutical developments. Regulatory agencies (FDA, EMA) strongly recommend and welcome new drug applications that include QbD aspects *(FDA, 2012; EMA, 2012*). Steps of QbD based development in pharmaceutical technology include the following:

1. Definition of Target Product Profile (TPP) and its quality indicators (Quality Target Product Profile, QTPP). This usually comprises therapeutic requirements and other quality demands (*EMA/CHMP, 2014*).

2. Identification of Critical Quality Attributes (CQAs) and Critical Process Parameters (CPPs), which have critical influence on the desired final product quality. CQAs are generally associated with the drug substance, the excipients, the in-process materials or the drug product. CPPs are those process parameters which have an impact on the CQAs. The selection of the CQAs and the CPPs should be based on previous scientific experience and knowledge from relevant literature sources (*Yu et al., 2014*).

3. RA is a systematic process of organizing information to support a risk decision*(EMA, 2015*) and is the key activity of the QbD based methodology. RA can be both initial and final, and it may be refined afterwards. RA results help to avoid profitless efforts in later phases of the development process.

The entire QbD methodology, including its steps and elements, is presented graphically in **Fig. 2**. This graphical illustration is based on the relevant the International Council Harmonization (ICH) guideline (*EMEA/CHMP, 2009; EMEA/CHMP,1998*) and is completed by the authors with the "primary knowledge space development" section and the illustration of "knowledge/design and control space" relation to help the better understanding.

In pulmonary drug delivery the main objective of inhalation is to achieve a reproducible and high pulmonary deposition. This may be achieved by a successful selection of the composition and careful process optimization (*Ambrus et al., 2011; Arafa et al., 2007*). The aerosolization efficiency of a powder for inhalation is highly dependent on the DPI characteristics, such as particle size, distribution, shape, and surface properties (*Pomázi et al., 2014; Pomázi et al., 2013*). A new tendency in the development of DPI is the design of carrier-free microparticles with a particle size of 3-5 μm as pulmonary drug delivery systems involving different excipients and additives (*Singh et al., 2005*). The additives applied in small amounts in microparticles serve to improve physicochemical stability, wettability, dispersibility and

aerodynamic properties (*Vehring, 2008*). DPIs have special formulation and regulatory aspects and their design is a highly complex task (*Hoppentocht et al., 2014; de Boer et al., 2017*). The powder formula and the administration manner should be designed parallel, therefore DPI are defined as combined products.

A regulatory and QbD based DPI product development process has several parameters that need special attention and critical thinking (*EMA/CPMP, 1998; EMA/CHMP, 2006; FDA, 1994*). Usually these include the following: (1) Drug substance specifications (e.g. particle size, particle size distribution, shape, crystallinity etc.). (2) Moisture and temperature sensitivity aspects to avoid aggregation. (3) Specifications of the excipients. (4) Packaging (delivery device) for uniform dosing and for assuring the fine particle mass. The current research examines regulatory science, namely the QbD and RA based thinking in early stage pharmaceutical technological development of a DPI form for pulmonary use.

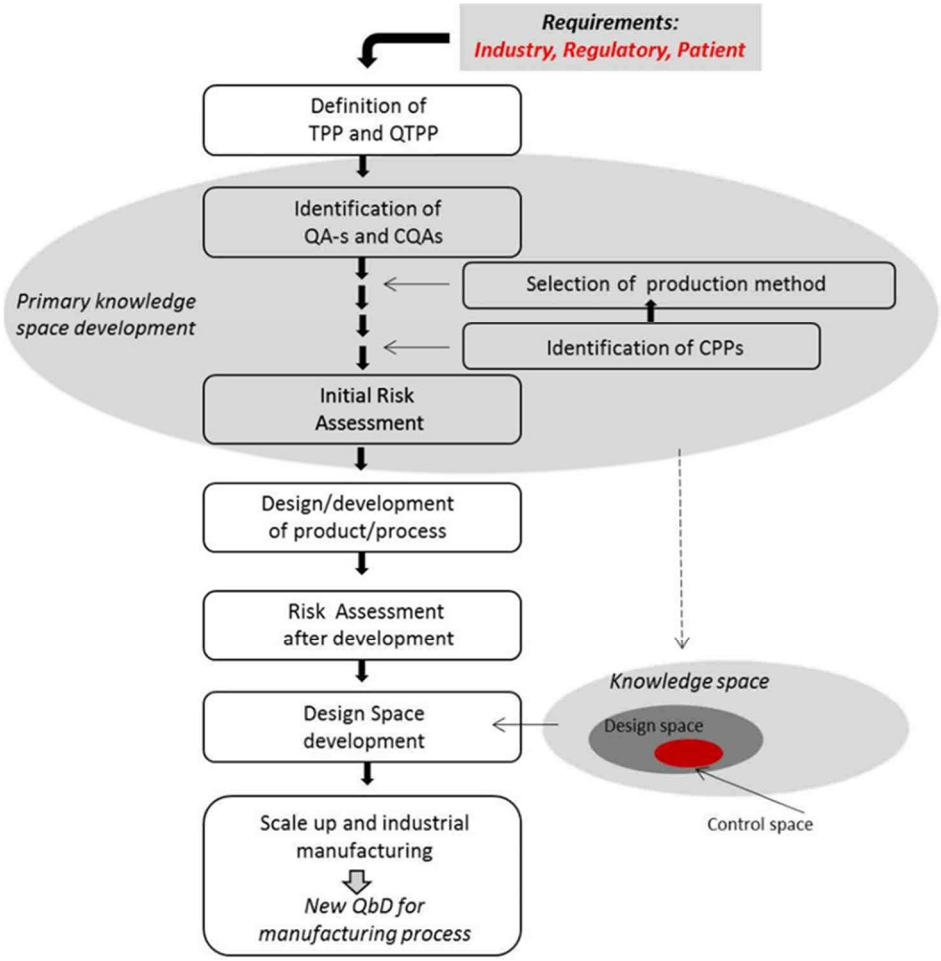


Figure 2: *Steps and elements of the QbD methodology completed by the authors and applied in the early stage of pharmaceutical development (Pallagi et al., 2016)*

1.5 DPI Formulation, stress and accelerated stability tests

One of the key factors involved in optimizing the DPI performance is the precision particle because it is one of the most important part of optimizing the DPI performance in order to obtain an accurate, consistent and effective drug doses powder formulation. Specifications for the approval requirements of new DPI is set by the FDA (*FDA, 1998*) and the European Inhalanda group (*Inhalanda, 1998*) and their test methods are equilibrated. The product must be in appropriate size aimed inhalation in order to be deposited in the respiratory tract. This depends on the characteristics of the product and must be assessed. In order to achieve reduced particle size to < 5 µm spray drying method has been frequently used *(Miller et al., 2016;* Sosnik and Seremeta, 2015*).* It is postulated that by manipulation of particle density to produce a mean aerodynamic diameter of 1-5 µm (density of < 0.4 g/cm3), the particles larger than 5 µm are not able to reach and presented to the alveolar space *(Telko and Hickey, 2005*). However, the presence of excipients is also a crucial factor in terms of modifying the surface and stabilizing particle size. L-leucine (LEU) an amino-acid, Hydroxypropyl beta cyclodextrin (CD), a cyclic oligosaccharide and Polyvinyl alcohol 3-88 (PVA), a synthetic polymer are frequently used as excipients in pulmonary drug delivery. LEU can enhance the aerosolization behavior of DPI due to decrease the dispersity of particles (*Prota et al., 2011*). CD can intensify the drug distribution in respiratory tract by cause of decreasing the cohesive and adhesive forces (*Pitha et al., 1986).* PVA can decrease the particle size as a result of covering effect of polymer (*Pomazi et al., 2013*). Using ethanol as co-solvent can help to produce micronized systems. Belotti et al concluded that the highest lung deposition was reached upon applying a maximum of 10% of ethanol beside the excipients (*Belotti et al., 2014*). Another objective when green technology is applied for drug formulation, chemical products and processes are designed, manufactured, and disposed with reduced environmental pollution risk and a lower burden of hazardous substances *(Fujii et al., 2016*). Green technology encompasses a group of methods and materials, which not generating toxic products. Hence in this field the invention, design and application of pharmaceutical products and processes is in direction to decrease or to eliminate the use and developing of hazardous substances (*Desal, 1981*).

Therefore, the goal of our study was to design a DPI of ciprofloxacin hydrochloride (CIP) in the form of a carrier-free system by applying green technology. The next step of our work was to test the stability of DPI in high humidity and temperature. In most of the reported studies, formulations were investigated to storage under high RH and temperature for only short periods of time (a maximum of 7 days).

1.6 Importance of stress and accelerated stability test

Stability testing is performed to confirm that drug products keep their entire efficacy up to the end of their expiration date, and a series of analytical investigations are necessary for this purpose *(Láng et al., 2015; Rabel et al., 1999; Bajaj et al., 2012)*. Test results relevant to the stability of the product must be in the predefined limits up till the end of the expiration date. FDA and the European Inhalanda Group have published their principles for the tests necessary for the approval of a new DPI.*(Department of Health and Human Services, 1998; Schumacher and Leiner, 2012 et al., 2012)*. The storage conditions during stability testing are set on the basis of the ICH (International Council on Harmonization) Guideline of Stability Testing of New Drug Substances and Products Q1A (R2).(*To et al., 2004)*. The ICH Guideline specifies the following storage conditions for stress and accelerated tests: 40 ± 2 ºC with 75 ± 5 % RH and the duration of storage is 6 months.

1.7 Cytotoxicity and permeability of DPI in epithelial lung cells

The cytotoxicity test is one of the biological assessments and screening tests that apply for living cells *in vivo* to detect the cell growth, reproduction and morphological effects by pharmaceutical products *(Suresh et al., 2012; Marslin et al., 2017)*. Cytotoxicity assays are extensively used by the pharmaceutical research to screen for cytotoxicity in studying of compounds. Considering of the sensitive respiratory mucosa and epithelial lung cells, the active ingredients likewise excipients need to be examined in appropriate models for collecting data about the safe concentration for pulmonary delivery (*Horváth et al., 2016*). Damage of lung epithelial cell will enhance with increasing concentration of product and key point of safety of product is that in applicable dose the concentration of active ingredient and excipients have to be in safe and normal range *(De Barros et al., 2017; Krátký et al., 2017)* (**Fig. 3**).

Besides active agent excipients can damage epithelial lung cells and during their application toxicity often occur. Despite they are necessary for their transport across biological barrier (*Deli, 2009*). The epithelial barriers of the human body are considerable walls for drug delivery to the respiratory system (*Pohl et al., 2009*). In spite of this physical barrier, comprised of the mucociliary apparatus, secreted antimicrobial substances which helps to treat infection prevents inhaled antimicrobial drug contacting the subepithelial tissue (*Deli, 2009*). Hence, permeability of these barriers could significantly promote the efficacy of the products It is essential to prove the diffusion of drug into epithelial lung cells in order to continue the work and further

research *in vivo* for large scale up manufacturing in industry. There are several routes in these barriers, which drug permeability is achieved via them (**Fig. 4**).

- by diffusion along the phospholipid bilayer
- the transcellular pathways via integral and peripheral protein transporters
- the paracellular pathway

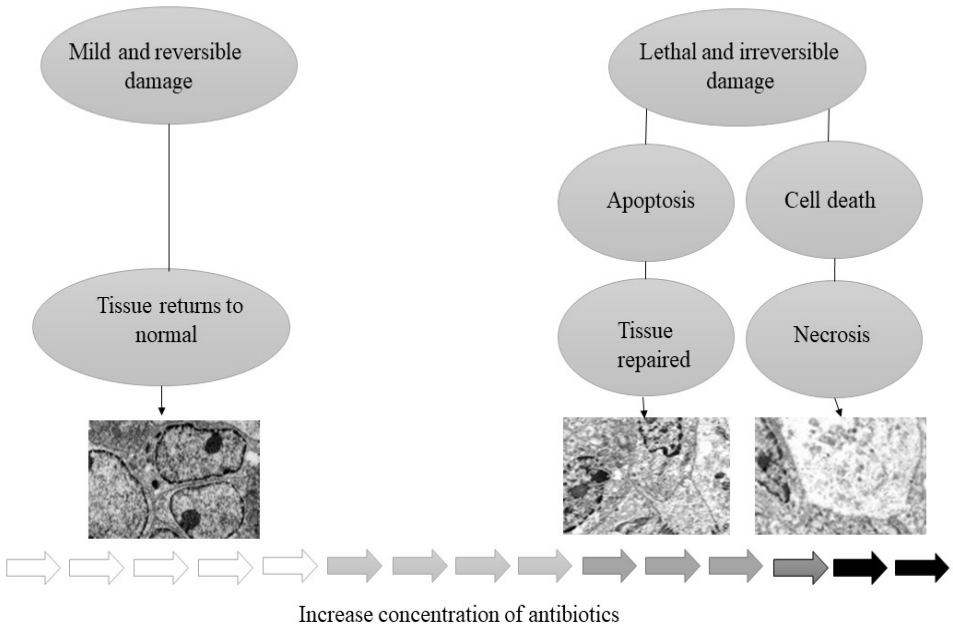


Figure 3: *Relationship between concentration of active agent and effect on living cells*

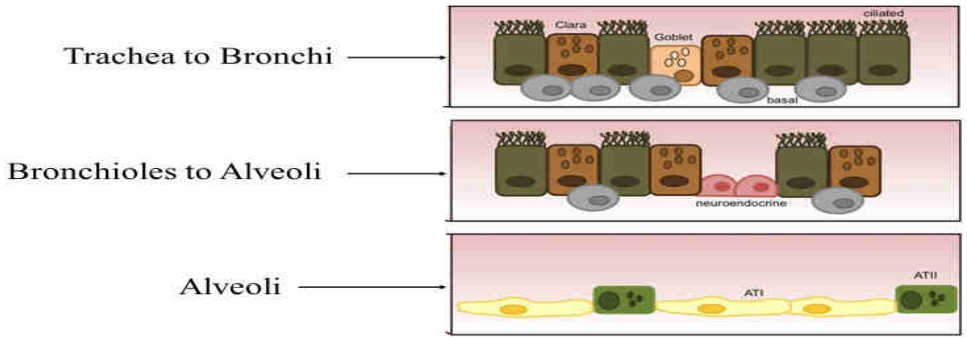


Figure 4: *Cells in different part of respiratory system including trachea, bronchi, bronchioles and alveoli (Camelo et al., 2014)*

2. AIMS

As the primary step of this study, the literature background and marketed products of DPI in antibiotics was collected. Secondly the introduction of the key elements of QbD approach to design dry powder for pulmonary delivery application – was set up. Based on these preliminary data, the next objective was to develop a carrier-free co-spray-dried DPI product containing the broadb spectrum antibiotic ciprofloxacin (CIP). CIP can be useful for treatment of variety of pulmonary infection disease such as those in patients with CF. This development is a novelty as QbD approach was applied for designing the experimental part and the development holds the possibility of large scale up manufacturing in industry. The design of carrier-free microparticles ("green technology") with large dose of medicine along with minimum amounts of excipients is also a new tendency in the development of DPIs. The additives are carried out in small-scale amounts in the microparticles in order to build up greater aerodynamic performance and physicochemical stability along with least possible cytotoxicity. The main steps in our experiments were the following:

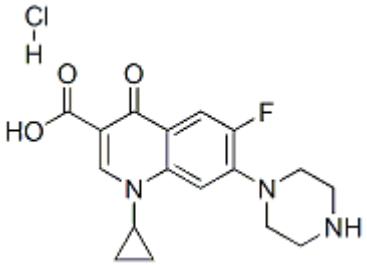
i. Pre-formulation: Identification of important factors for formulation of DPI based on carrier-free system by applying green technology based on the QbD approach. Focusing on the critical parameters, the practical development, connections and effects among the material characteristics, selected production process, investigation methods and final product properties in the design phase.
ii. Formulation: Study of different excipients (polymers, sugars and amino acid) to develop the pulmonary formulation with optimal size and superlative respirable fraction. Hence analysis the physiochemical properties of formulations. Afterwards investigation *in vitro* release of samples.
iii. Investigation of the stress and accelerated stability test of co-spray-dried products: Investigation influence of the relative humidity (RH) and temperature on the physicochemical properties and aerosolization performance of the formulations during storage.
iv. *Ex vivo*: Determination of the cytotoxicity of samples in epithelial lung cell line culture to screen the safety of formulation for pulmonary delivery along with investigation of permeability of formulation that one may to promote information on the availability in pulmonary formulations.

3. MATERIALS AND METHOD

3.1 Materials

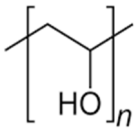
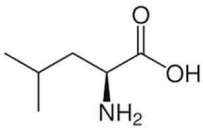
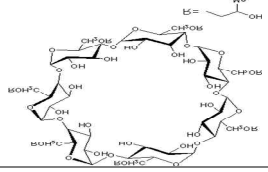
CIP, a fluoroquinolone-type antibiotic was supplied by Teva Pharmaceutical Works Ltd. (Debrecen, Hungary). CIP is a broad spectrum synthetic agent. Its main mechanism of action is the inhibition of the bacterial enzymes DNA gyrase (topoisomerase II) and topoisomerase IV, thus preventing bacterial DNA from uncoiling and duplicating, leading to cell death. It has potent and effective activity against a wide range of Gram-positive bacteria and against most Gram-negative microorganisms and it is often used in the treatment of inhalation anthrax and other lung infections. (**Table II)**.

Table II: *Properties of the active agent*

	Ciprofloxacin Hydrochloride
Chemical structure	
Chemical name	1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride
Physical properties	White to light yellowish powder substance
Classification	A second generations fluoroquinolone antibiotic
Dosage Forms	Oral, Parenteral
Dose	Oral: 500mg Parenteral: 400mg

Polyvinyl alcohol 3-88 (PVA), a water-soluble synthetic polymer as a coating material was purchased from BASF (Cologne, Germany)(*Satoh et al., 2014*). The amino acid L-Leucin (LEU) was obtained from Hungaropharma Ltd. (Budapest, Hungary). This amino acid can be co-spray-dried with active compounds to enhance drug aerosolization behavior (*Li et al., 2016).* Hydroxypropyl beta-cyclodextrin (CD), a cyclic oligosaccharide was donated by Cyclolab Ltd (*Pitha et al., 1986)* (Budapest, Hungary) (**Table III**).

Table III: *Properties and structure of the excipients*

Excipients	**Polyvinyl alcohol 3-88 (PVA)**	**l-leucine (LEU)**	**Hydroxypropyl beta-cyclodextrin (CD)**
Chemical structure			
Physical properties	a white powder semi-crystalline	a white crystalline powder	a white powder
Applications	a stabilizing agent a microfine coating material	a dispersity enhancer, an aerosolization performance promoter	drug distribution enhancer

3.2 Methods

3.2.1 QbD method

As part of the QbD methodology, knowledge space development and RA were performed. To illustrate the relevant knowledge and information, an Ishikawa diagram was set up. The technical tool used for the RA was LeanQbD® software (QbDWorks LLC, Fremont, CA, USA). In the first step, the desired product was defined and the elements of the quality target product profile (QTPP) were determined. Next, the critical quality attributes (CQAs) and the critical process parameters (CPPs) of the selected production method were identified. CQAs have critical influence on the quality characteristics of the final product and CPPs critically influence the CQAs and QTPPs. In the following step an interdependence rating was performed among the QTPPs and CQAs, and also among the CQAs and CPPs and was categorized on a three-level scale. The interaction between the elements was described as “high” (H), “medium,” (M) or “low” (L). Its dynamism is presented in figures generated by the software. This was followed by the probability rating step where CPPs were estimated and categorized on a 10-point scale. Finally, Pareto charts were generated, presenting the numeric data and the ranking of each CQA and CPP**.**

3.2.2 Solubility of CIP at different pH

Solubility tests of CIP were carried out at 25°C either in a buffer solution (pH =1.2, 3.5, 5.6, 6.8 and 7.4) or in distilled water (pH =4.4). Solubility was measured by ultraviolet/visible

spectroscopy (UV/VIS) spectrophotometry (ATI-UNICAM UV/VIS spectrophotometer, Cambridge, UK). The concentration was determined 24 h after filtering the saturated system.

3.2.3 Preparation of the microparticulate systems and process parameters

The significant solubility of CIP in distilled water allows its use as the solvent for spray-drying feed solution. Using 10% of ethanol in an aqueous solution is known to decrease the particle size because of its fast evaporation during spray drying. Therefore, the feed solution was prepared by dissolving 1 gram of CIP using different excipients at different concentrations in an aqueous solution containing 10% of ethanol (**Table IV**). According to literature background about the effects of organic solvent and additives on the habit (size and morphology) and aerosolization characteristics of DPI systems, the optimal excipient concentration is achieved as shown in Table IV.

Table IV: *Composition of the DPI products containing an optimal concentration of excipients*

No.	CIP [g]	LEU [g]	PVA [g]	CD [g]	Solvent [ml]
CIP	1	-	-	-	50
CIP_PVA	1	-	0.2	-	50
CIP_CD	1	-	-	0.9	50
CIP_LEU	1	0.4	-	-	50
CIP_LEU_PVA_CD	1	0.4	0.2	0.9	50

Spray-drying (SPD) is a one-step process through which it is possible to engineer and produce particles directly from solutions with a controlled technique. Hence, spray-drying was considered to be the appropriate technique to produce a dry powder for inhalation. The spray-drying process was carried out using a Büchi Mini Dryer B-191 (BÜCHI Labortechnik, Flawil, Switzerland); the parameters were optimized as shown in **Table V**.

The amount of dry powder yielded was determined between 65% and 70%. Generally during the spray-drying procedure nearly 30% of the sample could be lost. So our produced yield correlated with the normal sample production’s habit.

Table V: *Büchi Mini Dryer B-191 parameters for spray-drying procedure*

Inlet temperature [°C]	**Outlet temperature [°C]**	**Feed rate [ml min^{-1}]**	**Aspiration air [L h^{-1}]**	**Aspiration rate [L min^{-1}]**
130	75	5	600	0.065

3.2.4 Particle size analysis

Particle size distributions of the spray-dried powders were determined by laser scattering using Malvern apparatus (Malvern Mastersizer Scirocco 2000; Malvern Instruments Ltd., Worcestershire, UK). Air was used as the dispersion medium for the microparticles from the entrance to the sample cell. Approximately 500 mg of product was loaded into the feeder tray. The dispersion air pressure was fixed to 2.0 bar to determine even if particle attrition had occurred. Obscuration between 10.0% and 13.0% was carried out throughout the whole measurement duration. The particle size distribution was characterized by the D (0.1), D (0.5) and D (0.9) values and the specific surface area. Span values were calculated according to Eq. 4. A high Span value (> 1) means a broad particle size distribution.

$$Span = \frac{D\ (0.9)\ - D\ (0.1)}{D(\ 0.5)} \tag{4}$$

3.2.5 Scanning electron microscopy (SEM)

The morphology of CIP microparticles was investigated by scanning electron microscopy (Hitachi S4700; Hitachi Scientific Ltd., Tokyo, Japan) at 10 kV. The samples were gold-palladium coated (90 s) with a sputter coater (Bio-Rad SC 502; VG Microtech, Uckfield, UK) using an electric potential of 2.0 kV at 10 mA for 10 min. The air pressure was 1.3–13.0 mPa.

3.2.6 Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectra were recorded with an FT-IR spectrometer (Thermo Nicolet AVATAR 330; LabX Midland, ON, Canada) between 4,000 and 400 cm^{-1}, at an optical resolution of 4 cm^{-1}. The sample was mixed with 150 mg of dry KBr in an agate mortar and the mixture was pressed to obtain self-supporting disks at 10 tons.

3.2.7 X-ray powder diffraction (XRPD)

The crystal structure of spray-dried powders containing different excipients was characterized using an X-ray powder diffraction BRUKER D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany). The powder samples were loaded in contact with a plane quartz glass sample slide with an etched square and measured with a slit-detector Cu K λI radiation (λ = 1.5406 Å) source. Settings were as follow: the samples were scanned at 40 kV and 40 mA and the angular range was 3° to 40° 2θ, at a step time of 0.1 s and a step size of 0.007°. The crystallinity index (xc) values were calculated based on the Eq. 5 where A marks the area under the whole curve:

$$Xc = \frac{A\ crystalline}{A\ crystalline - A\ amorphous} \times 100 \quad (5)$$

3.2.8 Differential scanning calorimetry (DSC)

The thermal response of each product was measured using a DSC (Mettler Toledo TG 821e DSC Mettler Inc., Schwerzenbach, Switzerland). About 3–5 mg of powder was precisely weighed into DSC sample pans which were hermetically sealed and lid pierced. Each sample was on equilibrate for 10 min at ambient temperature before being heated to 400°C at a rate of 5°C/min. Data analysis was performed using the STAR^{e} software (Mettler Toledo Mettler Inc., Schwerzenbach, Switzerland).

3.2.9 Aerodynamic particle size analysis

Aerodynamic particle size distribution was determined using a seven-stage Andersen Cascade Impactor (Copley Scientific Ltd., Nottingham, UK). The flow rate was set to 60 $Lmin^{-1}$. During the process, the aerosol moved along seven stages and according to the diameter and density of the particles can be deponated and then it was washed by methanol/phosphate buffer (60/40 v/v %) to collect the deposited drug amount. All samples were investigated by UV/VIS spectrometry at 271 nm (ATI-UNICAM UV/VIS Spectrophotometer, Cambridge, UK).

The fine particle fraction (FPF) was established as the number of particles deposited at stage 2 and lower than 5 μm, divided by the total initial amount of the particles filled in the inhaler (10 mg). The mass median aerodynamic diameter (MMAD) was defined based on the graph as the particle size at which the line crossed the 50th percentile, indicating the particle diameter at which 50% of the aerosol particles by mass are larger and 50% are smaller. Drug-emitted dose

(ED), defined as the percentage of CIP exiting the DPI, was determined by subtracting the amount of CIP remaining in the DPI from the initial mass of CIP loaded. To determine the drug content, 10 mg of the ciprofloxacin-bearing spray-dried microparticles was dissolved in methanol/phosphate buffer (60/40 v/v %)) and analyzed by UV spectroscopy.

3.2.10 *In vitro* release

To check the difference in drug release between the prepared products, 10 mL of phosphate buffer (pH 7.4, as the pH in the lung) was used to suspend an equivalent of 50 mg of CIP content in all products. After 1, 2, 3, 4, and 5 min, sample was taken out, filtered, and the concentration measured by UV spectroscopy at a maximum wavelength of 271 nm.

3.2.11 Stress and accelerated stability testing

Stability tests were performed as recommended by the ICH Q1A Guideline named "(R2) - Stability Testing of New Drug Substances and Products". Stability testing was carried out in a Binder KBF 240 (Binder GmbH Tuttlingen, Germany) equipment, with a constant-climate chamber. An electronically controlled APT line preheating chamber and refrigerating system ensured temperature accuracy and reproducibility of the results in the temperature range between 10 and 70 °C and the RH range between 10 and 80 %. Accelerated testing was performed at 40 ± 2 ºC with 75 ± 5 % RH. Samples were stored in hard gelatin capsules (size 3) (Capsugel, Belgium) in open containers; the duration of storage was 6 months. Sampling was carried out after 0 and 10 days, and 1, 2, 3 and 6 months.

3.2.12 Cytotoxicity testing

For cell culture A549 cells (ATCC, USA), a human immortalized alveolar type II like lung epithelial cell line, were cultured. A549 cells (passage number ≤ 35) were grown in Dulbecco's modified Eagle medium supplemented with 10 % fetal bovine serum (FBS, Pan Biotech, Germany) and 50 µg/mL gentamicin, in a humidified incubator with 5% CO_2 at 37ºC.

Human endothelial cells derived from cord blood hematopoietic stem cells were cultured in endothelial medium (ECM-NG, Sciencell, Carlsbad, CA, USA) supplemented with 5% FBS, 1% endothelial cell growth supplement (ECGS, Sciencell, Carlsbad, CA, USA), 1% lipid supplement (100×, Life Technologies, USA), 550 nM hydrocortisone, 10 µM retinoic acid and 0.5% gentamycin in a humidified incubator with 5% CO_2 at 37ºC.

Kinetics of lung epithelial cell reaction to treatment was monitored by impedance measurement at 10 kHz (RTCA-SP instrument; ACEA Biosciences, San Diego, CA). Impedance measurement is a label-free, real time, noninvasive method, and correlates linearly with adherence, growth, number, and viability of cells (Bocsik et al., 2016). For background measurements 50 µL cell culture medium was added to the wells, then cells were seeded at a density of 5×10^3 cells/well to rat tail collagen coated 96-well plates with integrated gold electrodes (E-plate 96, ACEA Biosciences). Cells were cultured for 4 days and monitored every 5 min until the end of experiments. At the beginning of plateau phase of growth, cells were treated with ciproflaxacin (1, 10, 30, 100 and 300 µM) alone or its formulations prepared with leucine, cyclodextrin (CD) and polyvinyl alcohol (PVA) for 48 hours. Triton X-100 detergent (10 mg/mL) was used as a reference compound inducing cell toxicity. Cell index was defined as Rn-Rb at each time point of measurement, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone.

3.2.13 Permeability testing

For the permeability test lung epithelial cells were co-cultured with endothelial cells for ten days. Lung epithelial cells were seeded on the upper side of cell culture inserts (Transwell, 0.4 µm pore size, 1.1 cm^2 surface area, Corning Costar Co., MA, USA). coated with rat tail collagen. Endothelial cells were passaged to the bottom side of the inserts coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Both cells were cultured in endothelial medium.

The transepithelial electrical resistance (TEER), representing the permeability of tight junctions, was measured on the co-culture model regularly. TEER was measured by an EVOM Volt/Ohm Meter (World Precision Instruments, USA) combined with STX-2 electrodes and expressed relative to the surface area of the monolayers ($\Omega\ cm^2$). The resistance of cell-free inserts was subtracted from the measured values. Before the permeability test the TEER was $135 \pm 11\ \Omega\ cm^2$.

In the permeability assay cell culture medium was changed in the lower compartment of the inserts to 1500 µL Ringer-Hepes solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM d-glucose,10 mM Hepes, pH 7.4) supplemented with 0.1 % bovine serum albumin. In the upper compartment culture medium was replaced by 500 µl Ringer-Hepes solution containing 10 µM of ciprofloxacin alone or its formulations. To check the monolayer integrity three samples were treated with 10 µM FITC-dextran 4.4 kDa (FD4), a permeability

marker molecule. The culture plates were kept in a 37 ºC incubator with 5 % CO_2 for 30 min on a rocking platform. After incubation the concentration of ciprofloxacin in samples from the apical and basolateral compartments was determined by HPLC. The concentration of the marker molecule FD4 was determined by a fluorescence multi-well plate reader (Fluostar Optima, BMG, Labtechnologies, Germany; excitation wavelength: 485 nm, emission wavelength: 535 nm). The apparent permeability coefficients (P_{app}) were calculated as we described previously (*Kürti, et al 2012*). Briefly, cleared volume was calculated from the concentration difference of the tracer in the lower/basal compartment ($\Delta[C]_B$) after 30 min (t) and upper/apical compartments at 0 hour ($[C]_A$), the volume of the lower/basal compartment (V_B; 1.5 mL) and the surface area available for permeability (A; 1.1 cm^2) by the Eq. 6:

$$P_{app}\,(\text{cm/s}) = \frac{[C]_B \times V_B}{A \times [C]_A \times t} \qquad (6)$$

3.2.14 Statistical analysis

All measurements were completed in triplicate and values are reported as means ±S.D. unless otherwise noted. Statistical calculations were performed with the software Statistical for Microsoft Windows 7. To identify statistically significant differences, one-way ANOVA with t-test analysis was performed. Probability values of $p < 0.05$ were considered significant.

4. RESULTS

4.1 QbD methodology and pre-formulation studies of DPI Products

For pre-formulation firstly an Ishikawa (fishbone) diagram was set up including all the parameters influencing the desired DPI product containing CIP as the active agent. The parameters were ranked into four groups (**Fig. 5**), namely 1. material characteristics, 2. production method, 3. test methods, and 4. product characteristics.

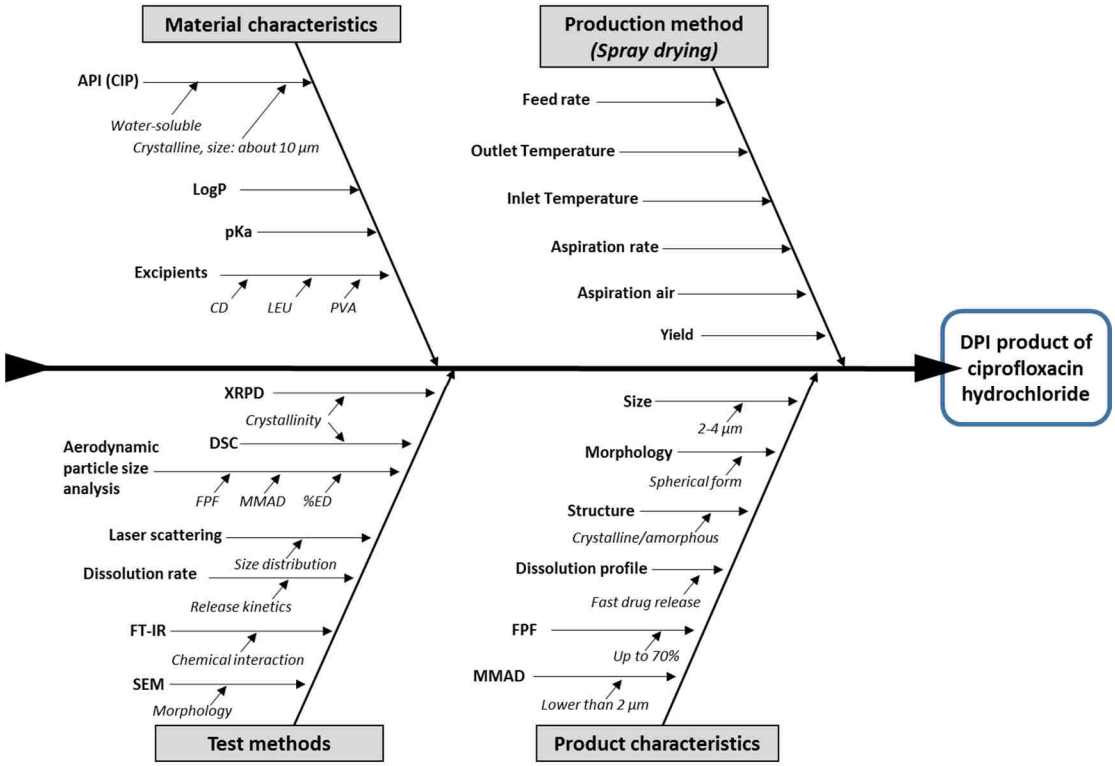


Figure 5: *Ishikawa diagram illustrating the parameters influencing the quality of the DPI product containing CIP (Karimi et al., 2016)*

This process served as a preliminary knowledge and information collection, which can help in the designing of the experiments and the selection of the CQAs and CPPs of the drug development procedure. The next step was the selection of the QTPPs, CQAs, and CPPs for the aimed DPI product. **Table VI** lists these with their selected targets and their justification, along with the explanations for the classification of the factors selected in each group.

Table VI: *Selected QTPPs, CQAs, and CPPs of a CIP containing DPI formula, their target, justification, and explanation*

	Target	Justification/explanation
QTPPs		
Therapeutic indication	Antibiotic (respiratory tract infections)	Antibacterial therapy is essential in respiratory tract infections. Ciprofloxacin is a second - generation fluoroquinolone antibiotic with a broad spectrum of activity. It is especially effective against infections caused by Gram-negative bacteria.
Target patient population	Adults	Literature data support a wide use of fluoroquinolone antibiotics in adults. It is a pregnancy category C antibiotic, considered to be safe during breastfeeding. It is also allowed for pediatric therapy but is never a first-line choice. Target patient group, as QTPP affects the dose selection and the route of administration.
Route of administration	Pulmonary administration	A relatively new route of administration for local antibacterial therapy. Pulmonary application avoids the first-pass effect, and reduces systemic exposure and the risk of side effects.
Site of activity	Local effect	Producing a local effect in pulmonary infections allows dose reduction of the antibiotics, associated with a lower risk of side effects. Pre-selection of the desired site of activity as QTPP affects the API used, as well as the dose and the dosage form selected.
Dosage form	Dry powder for pulmonary use	Dry powders with a particle size of 2-4 μm are required for the optimal deposition in lungs. Pre-determination of the adequate dosage form is a QTPP according to the ICH Q8 guideline.
Dissolution profile	Increased dissolution	Dissolution profile is a recommended QTPP as it affects the bioavailability and pharmacokinetics, and is critically related to the quality, safety and efficacy of the medicinal product.
CQAs		
Excipients (quality profile)	Excipients assure proper quality characteristics by modifying the size, morphology, hydrophility and stability of the DPI product.	Polyvinyl alcohol 3-88 (PVA) is a microfine coating material. The amino acid L-leucine (LEU) can be well co-spray-dried with certain active compounds to modify the drug's aerolization behaviour. Dymethyl-beta cyclodextrin (CD) can be applied as a drug distribution enhancer. As CQAs, excipients are critically related to the dissolution and quality profile of the final product.
Particle size /specific surface area (SSA)	Homogenous, microsized product of 2-4 μm	Microsize dimension has the optimal specific surface area and optimal administration properties for pulmonary use.

		Size is critically related to pulmonary administration and to its local and/or systemic therapeutic effect, thereby to product safety, efficacy and quality.
Appearance	Microparticle	Microparticle formulation is suitable to achieve an increased SSA, an improved wettability and a high amount of dissolved drug. It is critically related to efficacy.
Dissolution	Improved dissolution rate (100 % in 5 min)	The dissolution profile highly affects the therapeutic effect. Accelerated drug release induces an immediate local effect. It is related to the modification of the SSA, wettability and solubility.
Wettability	Hydrophile product	Wettability is critical for the drug's adhesion the pulmonary mucosa. It is critically related to efficacy.
Structure (cryst. /amorph.)	Stable form (cryst. /amorph)	The crystalline or the amorphous state of the API affects stability and release properties. It is critically related to efficacy and quality.
Solubility	Water-soluble	Solubility has a remarkable influence on the bioavailability of the drug/product.
CPPs		
Composition (*Co-spray drying)*	Micronized size, stabilized structure using additives.	Additives contribute to reaching the desired and pre-defined quality of the final co-spray-dried product.
Inlet temperature (*Co-spray drying*)	130°C	Inlet temperature has a critical influence on optimal drying and thus influences the final product's appearance.
Outlet temperature (*Co-spray drying*)	75°C	Outlet temperature has a critical influence on optimal drying of the desired co-spray dried final product.
Feed rate (*Co-spray drying*)	5 ml min^{-1}	Feed rate has a critical influence on the formation of co-micronized particles.

Figure 6 shows the elements and results of the QbD based RA. **Figure 6A** presents the interdependence rating of the QTPPs and CQAs, and of the CQAs and CPPs. Each interaction was ranked as high (H), medium (M), or low (L). The same three-level scale was used for the occurrence rating of the CPPs, which is presented in **Figure 6B**. These interdependence and occurrence ratings were followed by risk estimation calculations using the RA software, which produced a precise impact score (or severity score) for each critical influencing parameter. The calculated and ranked severity scores for the CQAs and CPPs are presented in Pareto charts (**Fig. 6C**) generated by the software. Pareto charts also give a graphical overview of the hierarchy of CQAs and CPPs based on their calculated numerical difference in their influence on the aimed quality of the product.

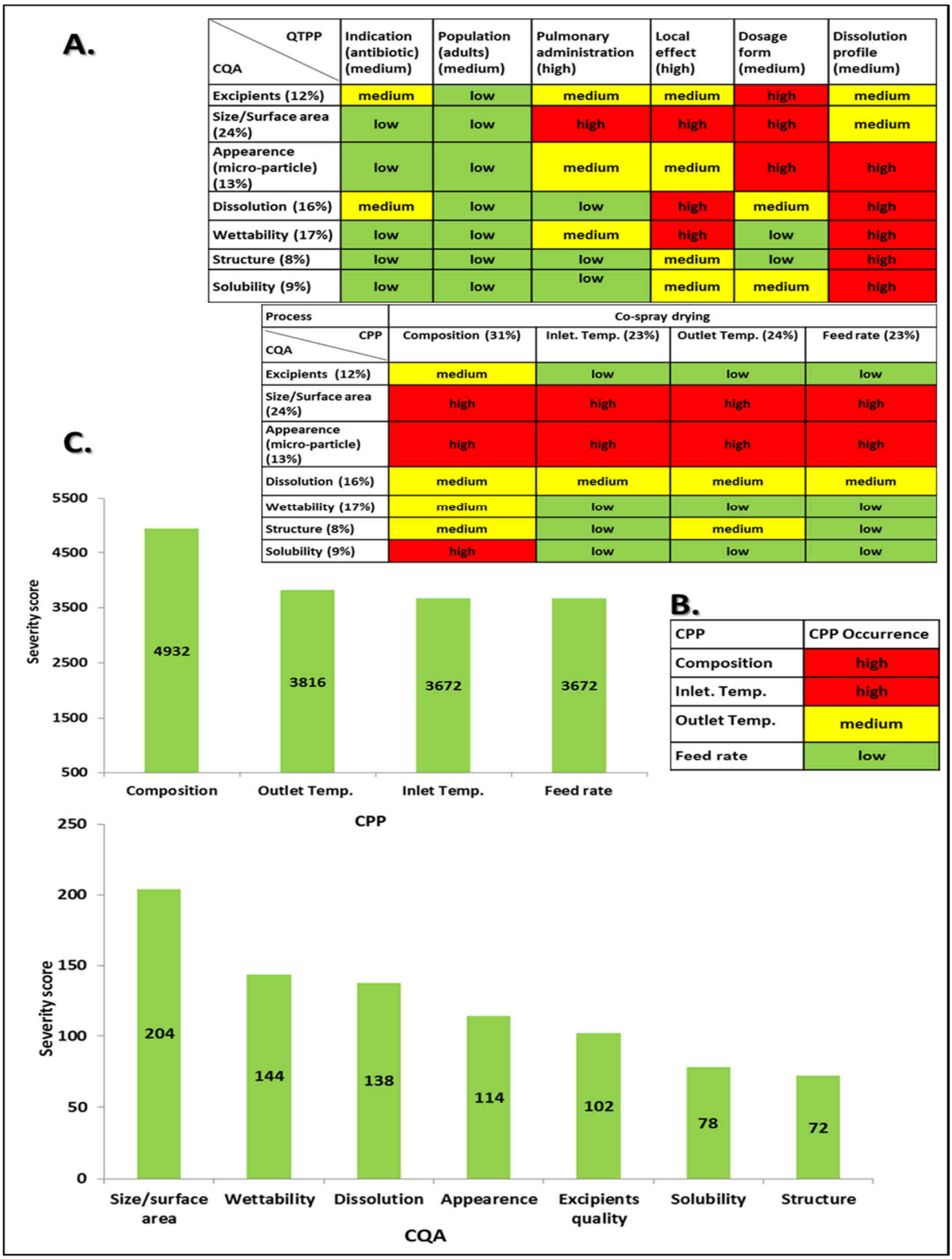


Figure 6: *Results of (A) the interdependence rating of the QTPPs and CQAs and of the CPPs and CQAs, (B) the occurrence rating of the CPPs and (C) Pareto charts of the CQAs and CPPs with calculated numeric severity scores generated by the RA software (Karimi et al., 2016)*

In this special case the particle size (or the specific surface area) of the API is the CQA to have the highest impact on the quality of the aimed final product. It is followed by wettability and dissolution properties. The next ones are appearance and the excipients' profile, while primer solubility and the structure of the API are found to have the lowest impact. The impact of "particle size and SSA" is 41.6% higher than that of "wettability" and is 47.8% and 78.9% higher than the impact of "dissolution" and "appearance", respectively. Comparing "structure" and "particle size", the difference in their impact on the quality of the final product is 183% for "particle size". Among the CPPs product, "composition" was found to have the highest impact on the desired final product's quality. It is followed by the "outlet temperature", then "inlet temperature", and "feed rate". The impact of the "composition on product quality" is 29% higher than that of "temperature". Nevertheless, there is a 34% difference between the critical process factor with the highest effect (composition) and the lowest effect (feed rate) on the final product's properties.

These results of the software-based RA highlight those factors that need the highest attention during the practical development phase when we decide about the exact composition and select the materials and excipients, etc. It has been established that the particle size of the API, the wettability and dissolution characteristics, as well as the composition of the DPI product are to be focused on during the practical development phase. The different DPI formulations were prepared according to these preliminary parameter rankings and priority classifying.

4.2 Characterization of formulated DPI containing ciprofloxacin hydrochloride

4.2.1 Solubility of CIP

Solubility test at different pH as a preliminary experiment was carried out. **Table VII** shows the solubility of ciprofloxacin hydrochloride at different pH and in distilled water. It is important to do this test to find out the optimal solubility and consequently the best solvent for CIP at different pH. According to the data shown in the **Table VII** the solubility of CIP is reducing in opposite direction to pH reduction. Remarkably, distilled water showed the best solubility for CIP.

***Table VII**: Solubility of ciprofloxacin hydrochloride in different pH and distilled water.*

Solvent	pH	Concentration [mg/ml]	λ max [nm]
Buffer solution	1.2	12.6	277
Buffer solution	3.5	36.0	277
Buffer solution	5.6	13.3	275
Buffer solution	6.8	0.4	272
Buffer solution	7.4	6.3	271
Distilled water	4.4	41.9	275

4.2.2 Micrometric and morphological properties

Since all the powders were prepared under similar drying conditions, in the spray-drying process the final particle size distributions of the samples were comparable. The smallest particle size was measured for the DPI of CIP containing PVA due to covering effect of PVA when used as excipient, while the largest value was measured for the DPI of CIP containing PVA, LEU, and CD as excipients. This size interval should be optimal for lung deposition, so a local treatment of the respiratory tract can be achieved by any of the compositions tested. Microparticulate DPIs had a narrow particle size distribution which is highly advantageous for pulmonary DPI, because it enables the particles to potently target a specific lung region. In consequence, it enables a high deposition of drug particles. Results of the particle size analysis are shown in **Table VIII**.

***Table VIII**: Particle size of the microparticles of various compositions prepared*

Material	D (0.1) [µm]	D (0.5) µm	D (0.9) µm	Specific surface area [m^2/g]
CIP_SPD	1.31±0.01	2.44±0.02	4.44±0.01	2.76±0.03
CIP_PVA_SPD	1.22±0.04	2.38±0.03	4.57±0.03	2.85±0.02
CIP_CD_SPD	1.68±0.03	3.02±0.06	5.24±0.04	2.20±0.09
CIP_LEU_SPD	1.62±0.05	2.84±0.02	4.84±0.00	2.33±0.01
CIP_PVA_CD_LEU_SPD	1.58±0.06	3.27±0.01	6.42±0.02	2.14±0.02

Field-emission scanning electron micrographs of the spray-dried microparticles are shown in **Fig. 7**. The DPI of CIP containing PVA has a relatively smooth surface with spherical geometry in contrast to the DPI of CIP containing LEU which was found to be cavitated. Such deep cavities can also be observed in the DPI containing CD, but those cavities are less deep than those in the DPI of CIP containing LEU. The DPI of CIP containing PVA, LEU and CD contains no cavities, which may result from the dominant effect of PVA to produce smooth surfaces.

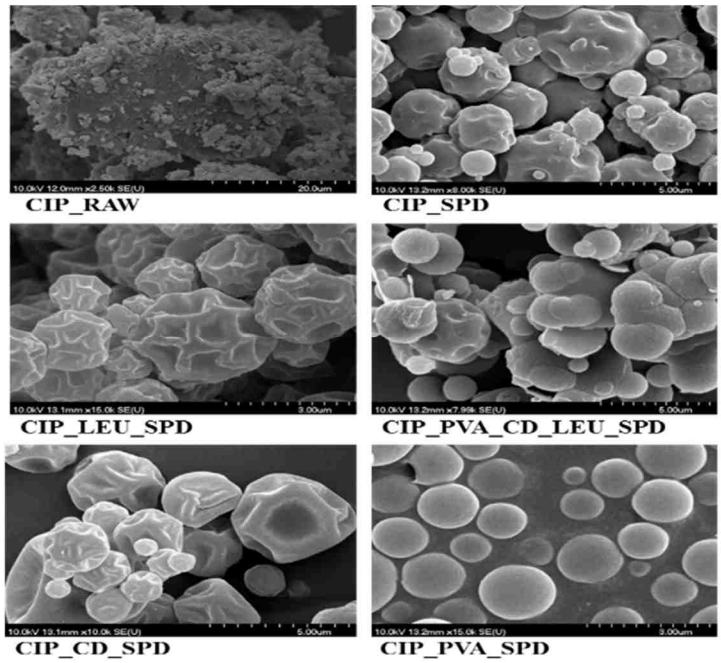


Figure 7: *Scanning electron micrographs of the spray-dried microparticles (Karimi et al., 2016)*

4.2.3 Structural characteristics

A sample containing only raw CIP was prepared by the same method to serve as a reference. The thermal response of CIP alone had a broad diffuse endothermic peak at about 145°C indicative of recrystallization and a sharp endothermic peak at 312°C indicative of melting.

In the thermograms of microparticles containing CIP and PVA, characteristic peaks were detected. There were two exothermic peaks at 152°C and 190°C due to recrystallization during spray-drying, and a sharp endothermic peak was detected at 312°C indicative of melting. Interestingly, the microparticles containing CD were shown to have a lower melting point at 207°C due to their amorphous structure resulting from spray-drying. The microparticles containing LEU were shown to have two different endothermic peaks at 270°C and 308°C indicating the melting points of LEU and CIP, respectively. **Fig. 8** shows the DSC thermograms

of raw CIP and of the different CIP-containing microparticles investigated.

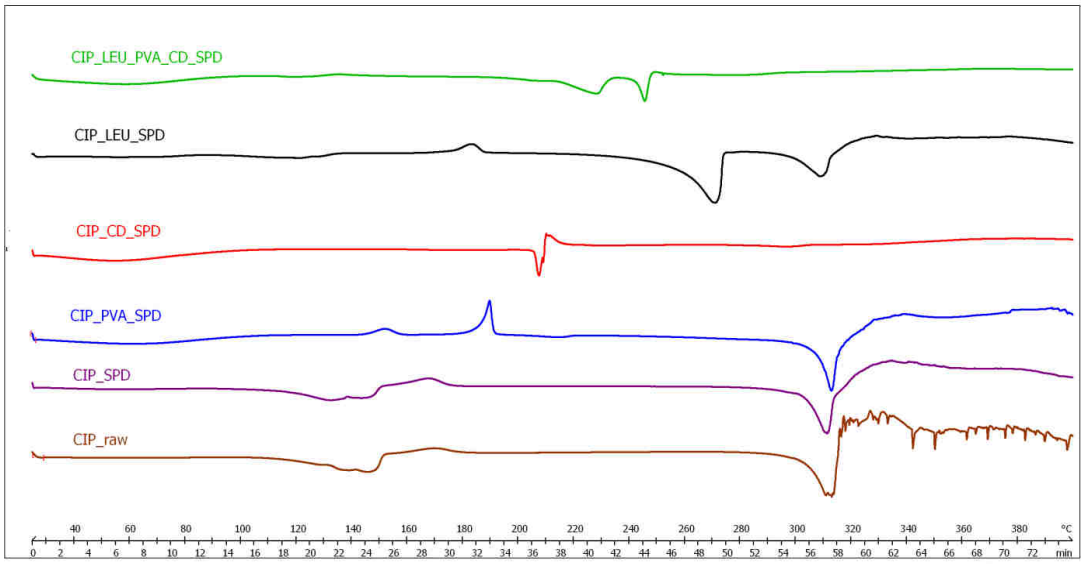


***Figure 8**: Thermograms of raw CIP and of the different CIP-containing microparticles investigated (Karimi et al., 2016)*

The XRPD diffractogram of raw CIP shows many characteristic peaks indicating a high degree of crystallinity, but the peak with the highest intensity is at 9.09° 2θ area.

Since the spray-drying parameters were similar for all the formulations, the differences in crystallinity must be attributable to the presence of the different excipients. The spray-dried products containing excipients exhibit a large degree of amorphicity and a very low degree of crystallinity. The only product that shows significant crystallinity is the DPI of CIP containing LEU, suggesting that the co-spray dried formulation is composed of crystalline LEU and amorphous CIP. Formulations containing CD have a completely amorphous character. The characteristic peaks of spray-dried microparticles at 6 2-Theta degree show the tendency of CIP to recrystallize from its polymorph.

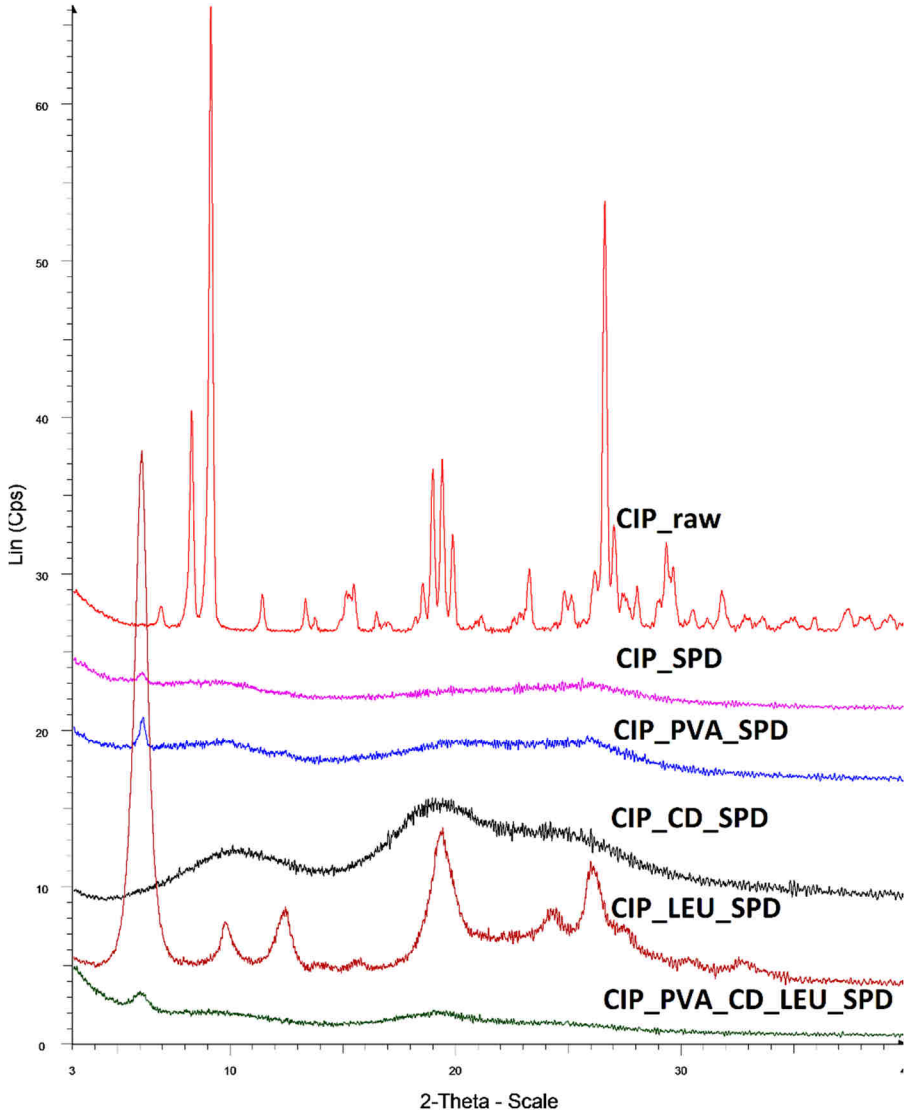


Figure 9: *XRPD diffractograms of raw CIP and of the CIP-containing microparticle compositions investigated (Karimi et al., 2016)*

The FT-IR spectrum revealed that raw CIP exhibits a peak at 1707 cm^{-1} as a consequence of the cyclic C=O stretching that induces a shift towards the higher wave numbers due to the association with the OH groups of other components. The magnitude of the shift in the C=O stretching for the bounded dimer form is inversely proportional to the strength of the interaction between CIP and the excipients. The other feature detectable at the FT-IR spectrum is the widening of the C=O stretching bond, caused by the configuration of the hydrogen realignment between the excipients and the C=O bond of ciprofloxacin. The shift of the above bond indicates that the interaction between CIP and LEU or PVA is weaker than that between CIP and CD. The bands assigned to N-H in the FT-IR spectrum are seen at 1612 cm^{-1}. For the microparticles containing excipients several peaks are missing. The peaks at 987cm^{-1} have completely disappeared in all cases due to the change from the dimeric to the monomeric state.

The bands assigned to N-H in the FT-IR spectrum can be seen at 1612 cm^{-1} both in the raw CIP and in the spray-dried microparticle compositions. **Fig. 10** Shows the FT-IR spectra of the raw material and the microparticle compositions investigated.

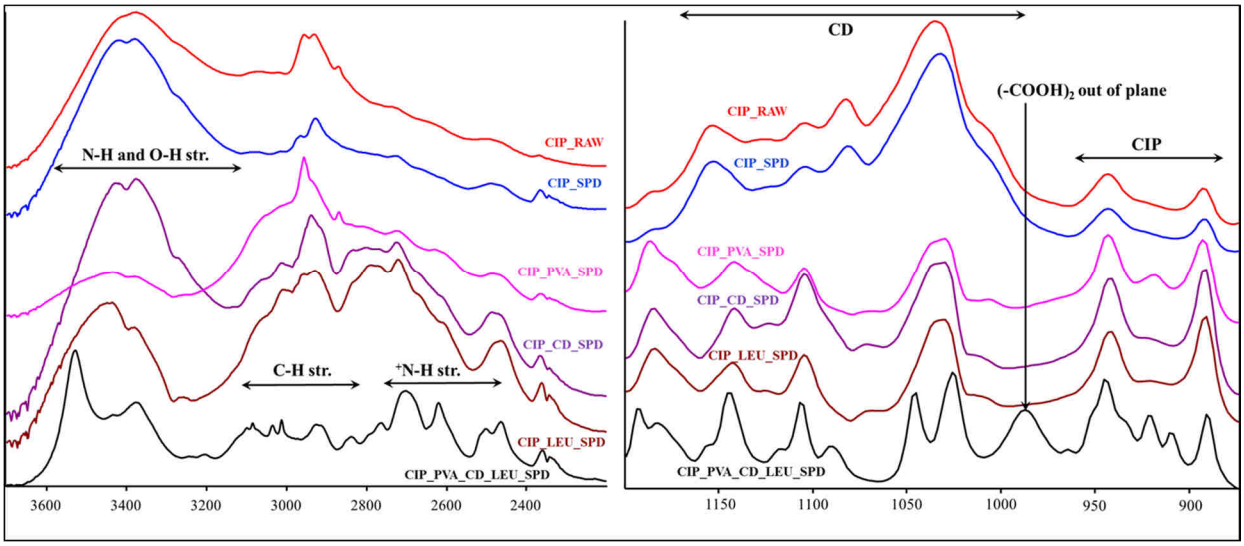


Figure 10: *FT-IR spectra of the raw material and the microparticles (Karimi et al., 2016)*

4.2.4 *In vitro* characteristics

Generally, the single CIP_SPD formulation had a low aerosol performance (FPF=31.68%±1.43). In contrast, the combined spray-dried formulations containing CIP plus excipients exhibited a significantly improved aerosol performance. While even the presence of PVA or CD raised the FPF value of the microparticles, those containing LEU were characterized by the highest aerosol performance. These results show that the use of excipients can significantly improve the aerodynamic behaviour of CIP-containing microparticles. LEU has the highest potential to improve the aerosol performance FPF=80.27%±1.65). This value indicates that more than 80% of the particles delivered from the inhaler have a volume aerodynamic diameter less than 5 µm. Drug contents, FPF, MMAD and ED values are shown in **Table IX**.

Dissolution tests focused on free CIP release from the samples. **Fig. 11** shows ciprofloxacin release from the microparticles at pH 7.4. A slower rate of CIP release was observed for the spray-dried microparticles containing PVA, compared to those without PVA. This behaviour can be interpreted by the controlled release induced by PVA. Drug release was shown to rise substantially in the presence of LEU and CD, and microparticles containing LEU as the only excipient were shown to be characterized by the highest rate of CIP release, with full API release within three minutes.

***Table IX**: Drug content, fine particle fraction (FPF), mass median aerodynamic diameter (MMAD) and emitted dose (ED) of the CIP-containing microparticle compositions investigated*

DPI	Drug content (%)	FPF (%)	MMAD (µm)	ED (%)
CIP_SPD	100%±0	31.68%±1.4	7.23±0.01	99.95%±0.5
CIP_PVA_SPD	85%±0.07	60.18.%±1.3	3.61±0.05	95.92%±0.4
CIP_CD_SPD	59%±0.03	58.54%±1.1	3.19±0.01	96.93%±0.5
CIP_LEU_SPD	72%±0.08	80.27%±1.7	2.15±0.08	95.81%±0.6
CIP_PVA_CD_LEU_SPD	45.%±0.24	45.93%±1.4	4.53±0.02	97.68%±0.4

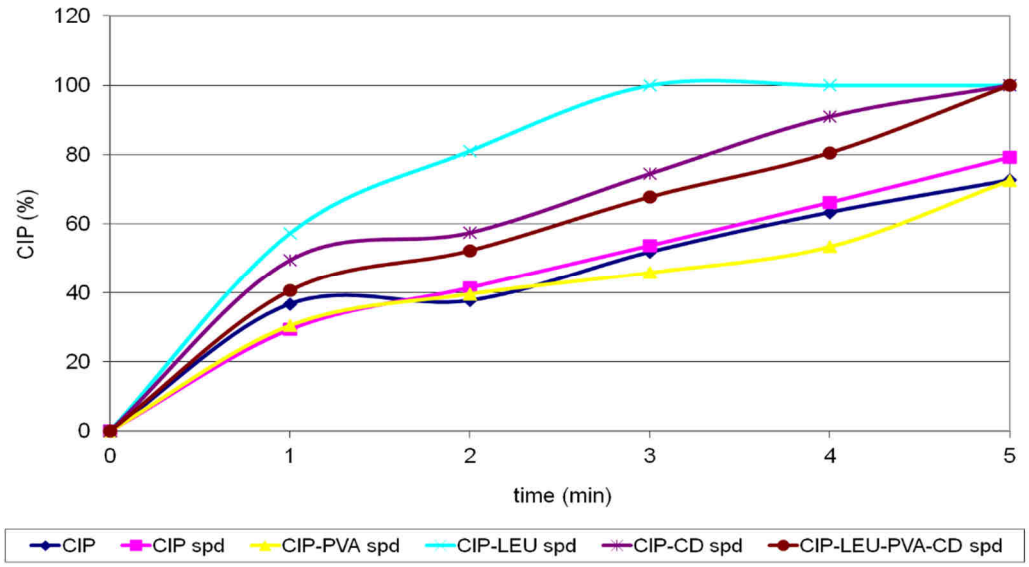


***Figure 11.** Kinetic plots of free ciprofloxacin release from the microparticles investigated (Karimi et al., 2016)*

The different DPI formulations were prepared, and all the particles produced are in acceptable particle size for optimal deposition in the lungs. Using PVA as an excipient for the CIP-containing DPI was shown to decrease the size of the microparticles significantly. The application of LEU to the microparticles induced a significant improvement in the FPF and in the aerodynamic behavior, and also allows a very fast drug release. Besides the adequate formula development, this study confirmed that all the microparticles after spray-drying are in amorphous form and it is crucial to study of stability of them further in the process as we did that.

4.3 Physicochemical stability testing and influence of humidity and temperature on aerodynamic properties

4.3.1 Particle size and morphological analysis

From particle size analysis aspect, since all the products are in the optimal range of particle size for DPI systems (2-5 µm), the studying of particle size during storage is of great importance in order to detect which of the products keep the particle size in the optimal range until the end of the storage time. The average particle size of single spray-dried CIP during storage ranged from 2.44 µm to 2.54 µm and did notshow significant difference during storage. Specifically, particles in this size range are able to adequately target and reach the deep bronchiolar and alveolar regions. The mean diameter of co-spray-dried CIP particles with LEU during storage ranged from 2.84 µm to 2.89 µm, which is suitable for targeted inhalation delivery as dry powders. In general, after storage median volume diameters of 20.78 ± 0.1 µm were observed for the spray-dried CIP with PVA, while values of 51.08 ± 0.1 µm were observed for samples containing CD. It means that while the polymer chains interact, they can lead to a reduction in physiochemical stabilization due to various potential interactions of the polymer chains and their ability to accept diverse conformations. Furthermore, CD can lead to an increase in particle size and the agglomeration of particles by reason of complex formation. The results also indicate that when the LEU is used in combination with CD or PVA, it exhibits a dominant effect and decreases the agglomeration of microparticles, but CD or PVA alone exhibits agglomeration and increases the size of microparticles greatly. The results of the particle size analysis (D 0.5) are shown in **Table X.**

Table X: *Average particle size of the microparticles of various compositions before and during storage*

Samples	Before storage [µm]	10 days [µm]	1 month [µm]	3 months [µm]	6 months [µm]
CIP_SPD	2.44±0.02	2.04±0.03	2.52±0.01	2.57±0.1	2.53±0.6
CIP_PVA_SPD	2.38±0.03	10.05±0.1	12.15±0.09	15.76±0.9	20.78±0.1
CIP_LEU_SPD	2.84±0.02	2.82±0.01	2.82±0.04	2.88±0.02	2.89±0.01
CIP_CD_SPD	3.02±0.06	3.32±0.02	6.22±0.03	7.73±0.05	51.20±0.1
CIP_PVA_CD_LEU_SPD	3.27±0.01	3.35±0.02	3.36±0.03	3.39±0.02	6.02±0.01

From particle form morphology perspective, a representative SEM image of the microparticles is shown in **Fig. 12**. In general, spray-dried microparticles with PVA have a relatively smooth surface with spherical geometry, typical of these excipients. During storage this morphology is retained but the agglomeration of particles can be observed. This agglomeration can also be seen in the microparticles which contain CD, but it is not as substantial as in the case of microparticles containing PVA. The use of LEU shows cavitated morphology. The stored products maintained the cavitated form, no changes in surface area and aggregation were detected. LEU stabilized the morphology and did not undergo any RH-induced changes, therefore the taking up of moisture during storage was prevented. The microparticles containing CD, LEU and PVA did not exhibit particle aggregation but the microparticles could not keep their spherical morphology, which is favorable for the aerodynamic behavior of DPI.

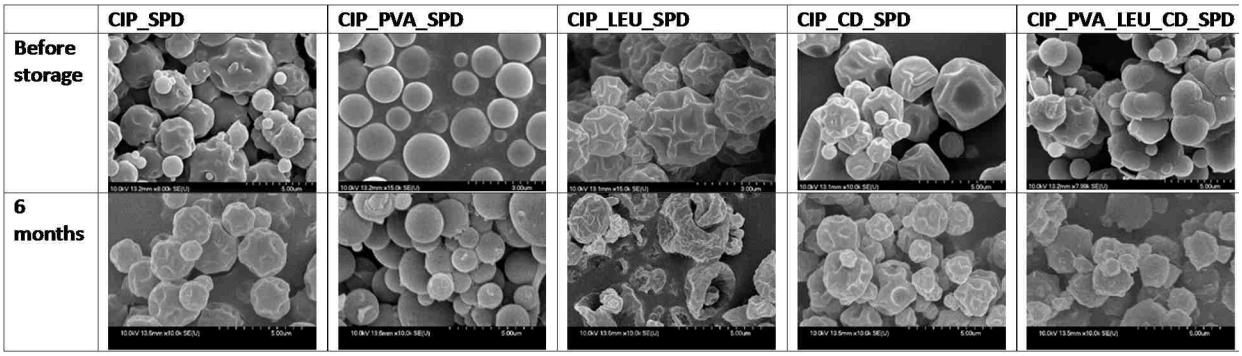


Figure 12: *Morphology of the prepared spray-dried samples during the storage (Karimi et al., 2018)*

4.3.2 Structural characterization

From structural characterization point by XRPD analysis of the samples according to the Cambridge Crystallographic Database, there is one crystalline structure of CIP and there are two existing polymorphs of ciprofloxacin base, the hydrate and hexahydrate forms. In order to investigate the crystallinity, amorphicity and polymorph form of the stored compositions, the X-ray powder diffraction method was used. The applied excipients (which are semi crystalline or amorphous) and spray-drying could affect the physicochemical properties of CIP. The XRPD patterns of the single spray-dried and co-spray-dried powders before and after storage are shown in **Fig. 13** and the crystallinity fractions of microparticles before and after storage are presented in the **Table XI**. The XRPD diffractogram of spray-dried CIP shows that amorphization took place in the sample. After 10 days of storage, the recrystallization of the

amorphous form began. By evaluating the diffractograms it can be observed that the amorphous microparticles recrystallized to CIP, and during storage no polymorphic changes occurred. The crystallinity fraction of the sample before storage was 5.49%, but after 6 months of storage it reached 95.76%. This significant and noticeable increase in the crystallinity fraction means that CIP has a tendency to fast recrystallization without any crystallinity inhibitor excipient during storage. The diffractogram of the sample containing PVA and CIP shows that spray-drying resulted in amorphization, but under storage ciprofloxacin recrystallized into its starting hydrochloride form. The tendency to recrystallization was lower than without excipient, and after 6 months the degree of crystallinity was only 55.81%. Microparticles containing LEU as excipient were mostly in amorphous form after spray-drying. After 10 days of storage, the characteristic peak of LEU appeared at 5.97° 2θ and the recrystallization of CIP was detected, which increased in samples stored for 1, 3 and 6 months and resulted in 98.28% crystalline fraction. The microparticles containing CD exhibited amorphization after spray-drying. CD protected CIP from fast recrystallization, after 6 months the crystalline content was only 41.50%. The samples containing PVA, LEU, CD and CIP were amorphous and the tendency to recrystallization decreased, only 39.65% crystalline fraction was detected in the sample after 6 months.

Table XI: *Crystallinity fraction of microparticles of different products before and after storage*

Crystallinity fraction	before storage	10 days	1 month	3 months	6 months
CIP_SPD	5.49%	65.17%	90.04%	90.36%	95.76%
CIP_PVA_SPD	11.38%	29.23%	33.86%	80.20%	95.81%
CIP_LEU_SPD	21.52%	36.61%	55.50%	82.50%	98.28%
CIP_CD_SPD	6.30%	7.99%	25.88%	54.57%	81.50%
CIP_PVA_CD_LEU_SPD	17.75%	18.38%	25.65%	32.76%	39.65%

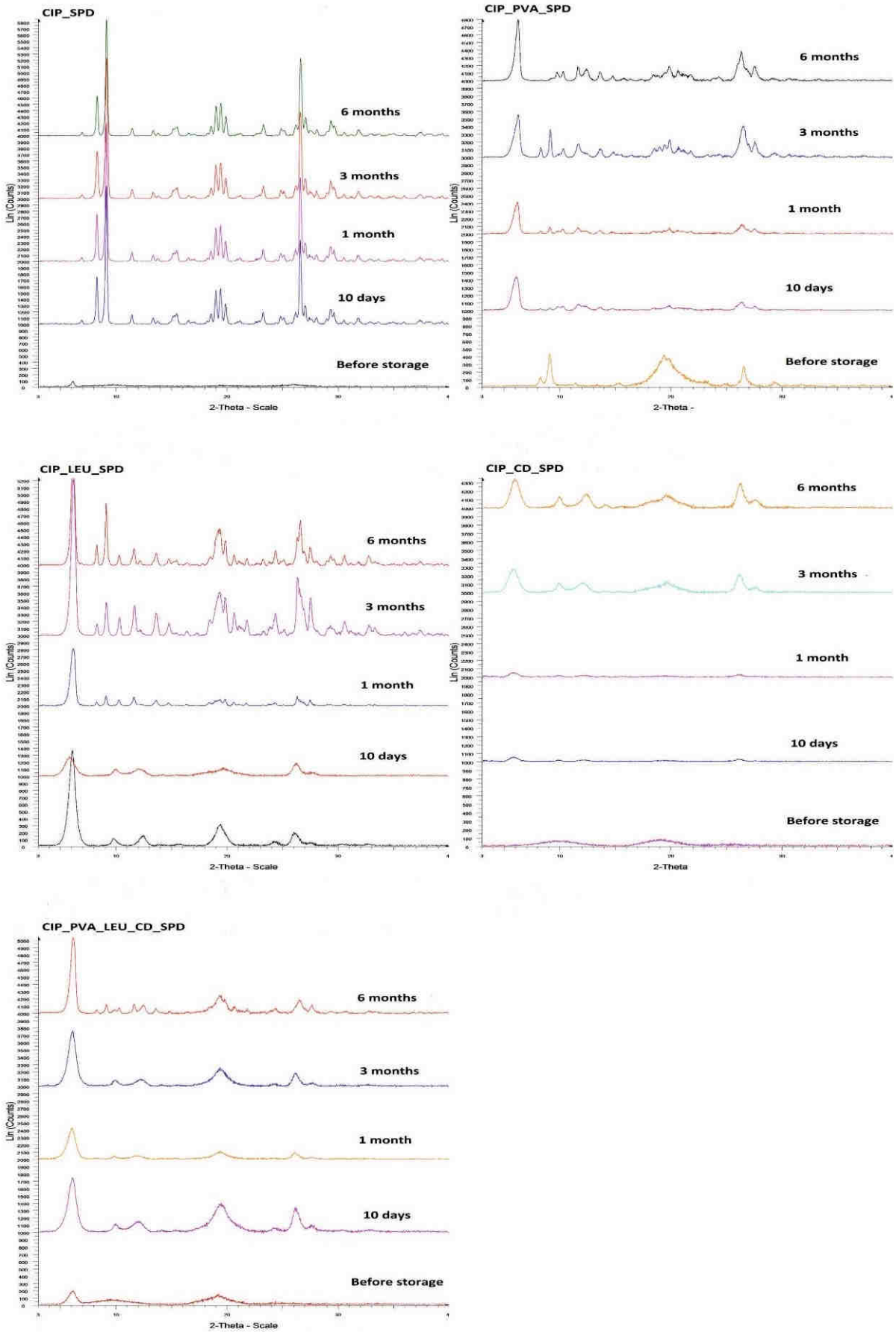


Figure 13: *Structural investigation of the products by XRPD before and after storage*

4.3.3 Thermoanalytical measurements

From thermo analytical measurements three polymorphic forms of CIP are reported in the literature by *Kakkar et al 1997*, Form I (Tm=313.5°C), Form II, (Tm=312°C) and Form III (Tm=316.3°C) [34]. DSC experiments were carried out to investigate the thermal behavior of CIP containing microparticles before and during storage (Fig 3.). When analyzing the thermogram of the single spray-dried CIP, the starting melting point of CIP at 311.05°C and the glass transition temperature at 149.93°C can be detected. Altogether it means spray-drying caused amorphization in the sample but some crystallinity fraction was preserved, which melted at 311.05°C. Consequently, after 1, 3 and 6 months the crystallinity fraction increased. According to the thermogram in **Fig. 14** of microparticles containing PVA, amorphization took place and the glass transition temperature can be observed at 151.01°C. This exothermic peak can be attributed to the recrystallization of amorphous ciprofloxacin at 190.02°C and this recrystallized CIP was melted at 312.55°C. However, glass transition after storage cannot be detected, which was in good agreement with increasing crystallinity in the compositions. Microparticles containing LEU exhibit glass transition at 158.82°C, LEU melts at 271.12°C, but the remaining crystallinity structure melts at 313.70°C. After 10 days, stored microparticles exhibited an exothermic peak at 182.86°C due to the recrystallization of CIP. The first endothermic peak at 270.67°C is the melting point of LEU and the other can be related to CIP at 309.72°C. Microparticles containing CD as excipient exhibit glass transition at 138.58°C. The melting of CD at 232.3°C was not observed in stored microparticles.

Microparticles containing PVA, LEU and CD have an exothermic peak at 202.3°C, where amorphous ciprofloxacin recrystallized into CIP II form, and an endothermic peak at 232.3°C where CD melts. The melting of CIP was not detected. In correlation with the XRPD results it was found that amorphous and crystalline form of CIP could be detected in the samples after storage.

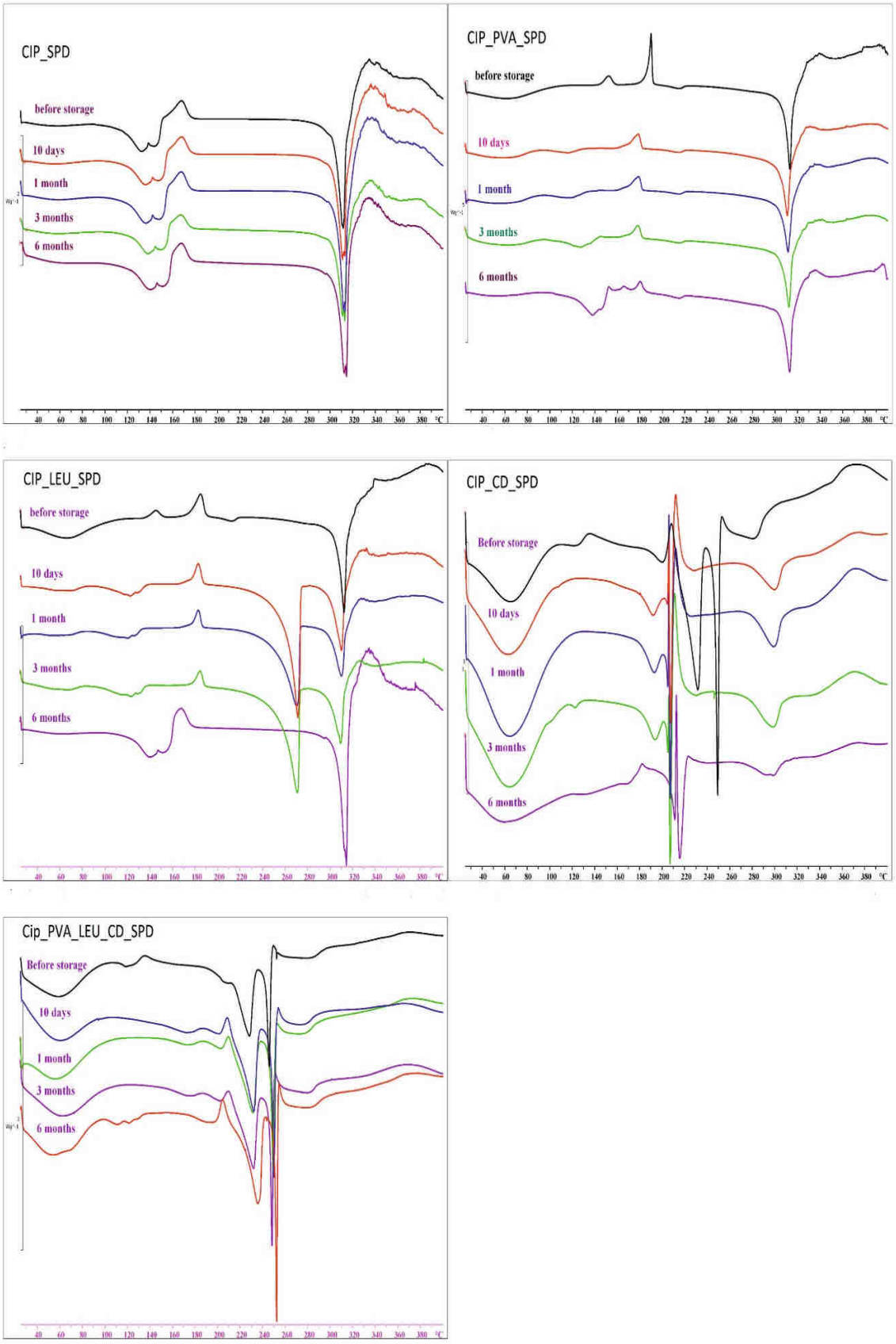


Figure 14: *Thermal analyses of fresh samples after 1, 3 and 6 months storage*

4.3.4 Aerodynamic particle size analysis

And finally, from most important property of DPI, aerodynamic particle size analysis of the microparticles were completed as established by means of the Pharmacopoeia test which is mentioned in detail in the Method part. To analyze the effects of excipients on the aerosol performance, the advancing deposition of the drugs was calculated. Single CIP_SPD showed a FPF value of 31.68 ± 1.4 %, which was enhanced during storage and reached the value of 67.35 ± 1.1 %. The MMAD of microparticles decreased from 7.23 ± 0.01 to 3.58 ± 0.03 after storage. Since single spray-dried CIP does not contain any excipient, it can be explained by the loss of water content and lowering the density of microparticles. In view of literature data, LEU is one of the excipients with the ability to enhance aerosol dispersibility, therefore before storage the FPF of microparticles containing LEU was 80.27 ± 1.7 % and the MMAD value was 3.52 ± 0.13 µm. However, in this experimental work our interest was focused on retaining high aerosol dispersibility during storage. After storage, the FPF value and the MMAD value of the stored formulations was 79.78±1.22 and 2.01±0.01µm, respectively; (**Table XII and Table XIII**). After storage, the FPF values of the samples did not change significantly. This is connected with retaining the particle size and morphology, as discussed before. This cavitated morphology and suitable particle size are favorable in terms of the aerosolization performance of the formulated DPI. Before storage, the FPF of microparticles containing CD was 58.54±1.1, although after storage the FPF values of the samples decreased because of the fine particle aggregation. This is associated with the increased particle size and retaining the amorphous structure for 3 months. Likewise, PVA did not have strong protecting effect in these formulations. As discussed in the previous part, this polymer agent induced aggregation in the formulation. Therefore, increasing particle size reduced the FPF of the PVA-containing microparticles to 25.00 ± 2.1%. CIP_PVA_CD_LEU_SPD containing microparticles did not keep the FPF value, which could be explained by the microparticles losing their spherical morphology. In conclusion it was found that soluble CIP consisting of PVA and CD's physiochemical property decline during storage. Aggregation was minimized in formulations with LEU and the other two formulations due to the stabilizing effect of LEU, and we continued the work (cytotoxicity and permeability test) with this three microparticles which exhibited acceptable aerosol performance, while maintaining the FPF during storage.

Table XII: *FPF value in % of microparticles before and after storage.*

Samples	before storage	10 days	1 months	3 months	6 months
CIP_SPD	31.68±1.4	52.98±1.9	54.96±1.4	64.60±2.1	67.35±1.1
CIP_PVA_SPD	60.18±1.3	56.90±1.5	34.47±2.0	30.86±1.1	25.00±2.1
CIP_LEU_SPD	80.27±1.7	84.71±1.0	80.21±2.1	77.31±1.9	79.78±1.2
CIP_CD_SPD	58.54±1.1	47.01±1.3	40.13±1.2	38.21±1.1	36.32±1.3
CIP_PVA_CD_LEU_SPD	45.93±1.4	48.00±1.1	39.17±1.9	39.00±1.0	28.28±2.3

Table XIII: *MMAD value in μm of microparticles before and after storage.*

Samples	before storage	10 days	1 months	3 months	6 months
CIP_SPD	7.23±0.01	4.52±0.02	4.31±0.04	3.62±0.01	3.58±0.03
CIP_PVA_SPD	3.61±0.05	4.10±0.04	7.57±0.03	8.4±0.01	11.18±0.02
CIP_LEU_SPD	2.15±0.08	2.59±0.02	2.15±0.06	2.67±0.03	2.01±0.01
CIP_CD_SPD	3.19±0.01	5.30±0.03	6.65±0.02	6.52±0.05	7.52±0.06
CIP_PVA_CD_LEU_SPD	4.53±0.02	5.47±0.05	6.55±0.03	6.98±0.02	9.11±0.07

This study presented the effects of different excipients (PVA, CD and LEU) during stability testing to find the appropriate excipients and stable microparticles to continue the work. Accordingly, it was found that CIP aggregation consisting of PVA and CD occurred during storage. However, this aggregation was minimized in formulations with LEU regardless of presence of other excipients, due to the stabilizing effect of LEU, and the powders exhibited acceptable aerosol performance. In conclusion, stability was improved by formulations with LEU.

It should be mentioned that we could also observe appropriate physio-chemical stability in the samples spray-dried without any excipients which could be explained by the increasing crystallinity.

4.4 Determination of cytotoxicity and permeability of spray-dried microparticles into epithelial lung cells

Following the physicochemical stability testing, it was shown that from five different types of microparticles three presented acceptable stability (CIP_SPD, CIP_LEU_SPD, CIP_LEU_PVA_CD_SPD). Thus, the work was proceeded with these three microparticles.

4.4.1 Cytotoxicity test

From cytotoxicity view microparticles contain CIP without excipient and CIP with LEU or combinations of excipients did not change the impedance of A549 lung epithelial cell monolayers in the range of 1-300 µM concentrations, indicating no cellular toxicity. **Fig. 15** illustrates Kinetics of lung epithelial cell reaction to treatment with ciprofloxacin at 1, 10, 30, 100 and 300 µM alone or its formulations prepared with LEU, CD and PVA for 48 hours.

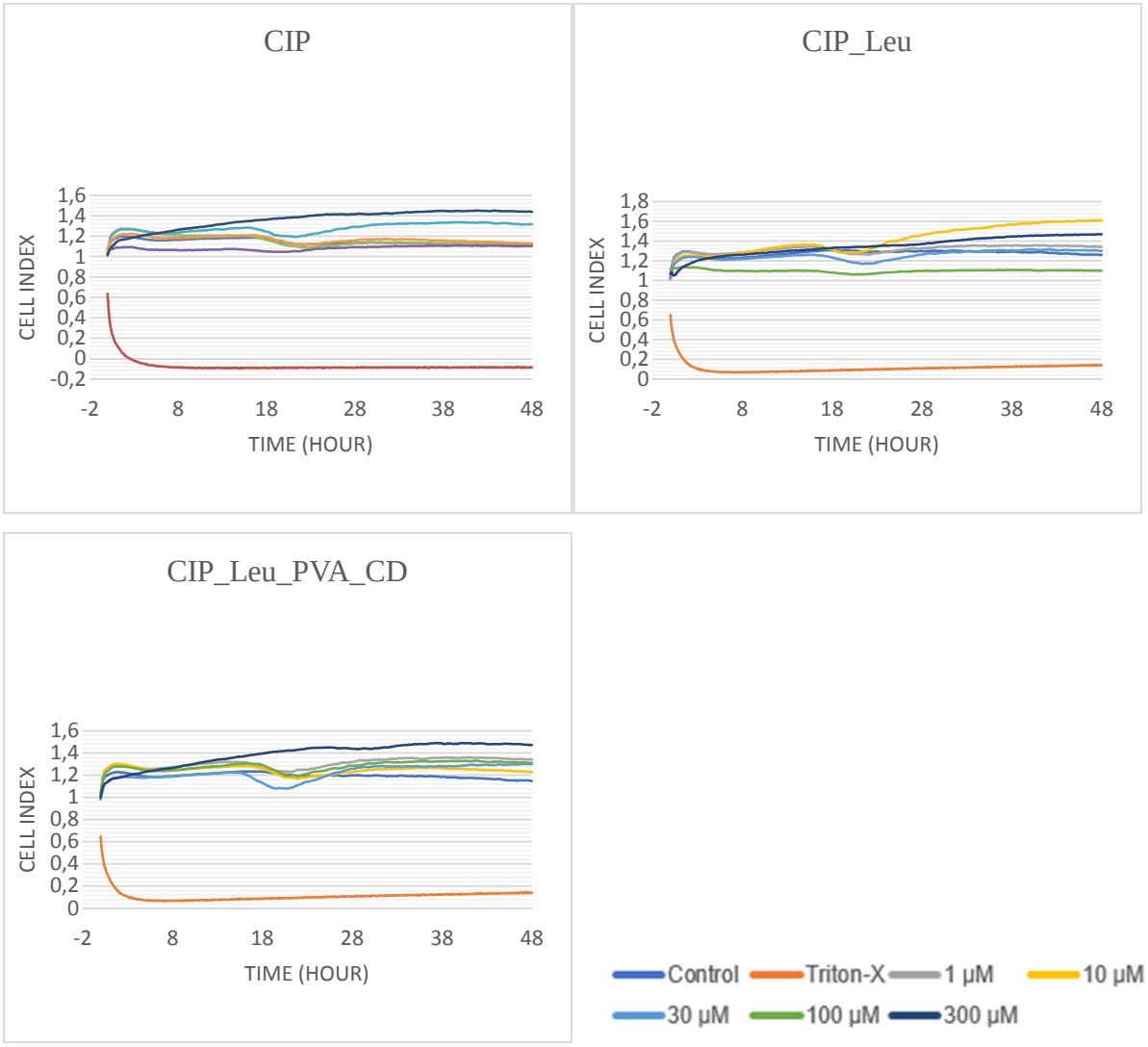


Figure 15: *Kinetics of lung epithelial cell reaction to treatment with CIP*

4.4.2 Permeability test

The TEER value of the co-culture model was 135 ± 11 Ω cm^2 before the permeability study and the P_{app} for the marker molecule, FD4 was 0.9 × 10^{-6} cm/s in concordance with previous results (Walter et al., 2016). No significant difference was found in the permeability value of ciprofloxacin compared to its formulation. **Fig. 16** explains permeability of CIP (10 µM, 30 min) and its formulation on a co-culture model of lung epithelium. FD4: FITC-dextran 4.4 kDa, permeability marker molecule. Values presented are means ± SEM. Statistical analysis: one-way analysis of variance followed by Bonferroni posttest. ***P < 0.001, compared to FD4 treated group, n = 4.

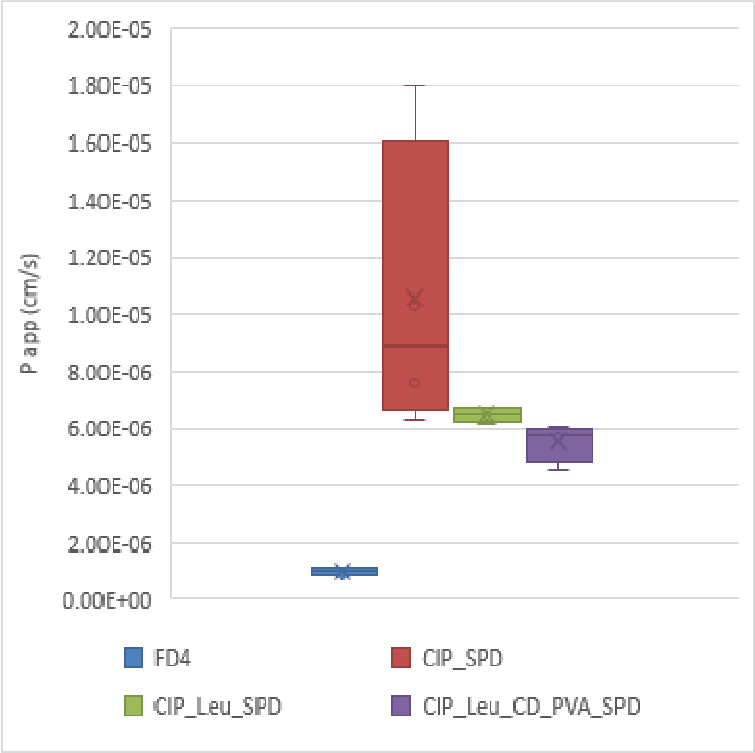


Figure 16: Permeability of CIP (10 µM, 30 min) and its formulation on a co-culture model of lung epithelium. FD4: FITC-dextran 4.4 kDa, permeability marker molecule. Values presented are means ± SEM. Statistical analysis: one-way analysis of variance followed by Bonferroni posttest. ***P < 0.001, compared to FD4 treated group, n = 4.

5. SUMMARY

The primary aim of this study was to carry out research relating to the formulation of DPI antibiotics. We studied the key features of QbD elements for use in pulmonary delivery system. Another objective was to develop a carrier-free, co-spray-dried DPI product containing high soluble CIP.

i. Quality by design (QbD), an up-to-date regulatory-based quality management method, was used to predict the final quality of the product. Quality by design (QbD) is a holistic, systematic, risk, science and knowledge based method, focusing on extensive preliminary design in order to ensure the quality of medicinal products. The basis of this thinking is described in the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). It has been established that the particle size of the API, the wettability and dissolution characteristics, as well as the composition of the DPI product are to be focused on during the practical development phase. According to the QbD-based theoretical preliminary parameter ranking and priority classification, DPI formulation tests were successfully performed in practice. The different DPI formulations were prepared according to these preliminary parameter rankings and priority classifications.

ii. According to significant solubility of CIP in distilled water we decided to use it as the solvent for feeding solution Spray drying showed to be a trustworthy technique to produce CIP microparticle. Then dry powder formulations were prepared and examined by particle size analysis, scanning electron microscopy, Fourier-transform infrared spectroscopy, X-ray powder diffraction, differential scanning calorimetry, and in vitro drug release and aerodynamic particle size analyses were also performed. The different DPI formulations were prepared, and all the particles produced are in acceptable particle size for optimal deposition in the lungs. Using PVA as an excipient for the CIP-containing DPI was shown to decrease the size of the microparticles significantly. The application of LEU to the microparticles induced a significant improvement in the FPF and in the aerodynamic behavior, and also allows a very fast drug release. These formulations displayed an enhanced aerosol performance with fine particle fraction up to 80%.

iii. Besides the adequate formula development, this study confirmed that all the microparticles after spray-drying are in amorphous form and it is crucial to study of stability of them further in the process as we did that. The next step of this work was to test the stability of

co-spray dried CIP. Since the microparticles in the dry powder system do not contain any stabilizer, the effects of temperature and RH on the physicochemical properties and aerosolization behavior are investigated. Hence investigation of the role of excipients such as PVA, LEU and CD on physicochemical stability and aerosolization performance is essential element prior designing the final dosage form. Particle characterization and size measurement as the most important parameters in aerodynamic behavior were examined. The overall stability results (against RH and temperature) showed that microparticles containing CIP and LEU alone and in combination with the other excipients were more stable than those containing PVA or CD alone. In relation to fine particle fraction and mass median aerodynamic diameter (determining the aerosolization parameters), it was found that the particle size and particle shape did not show significant changes after the storage. Among the excipients LEU was found to have many advantages, including relatively simple formulation, enhanced aerosolization behavior, convenient portability and inherently improved stability.

iv. Following the physicochemical stability testing, it was shown that from five different types of microparticles three presented acceptable stability (CIP_SPD, CIP_LEU_SPD, CIP_LEU_PVA_CD_SPD). Thus, the work was proceeded with these three microparticles. For cytotoxicity test firstly cell culture human immortalized alveolar type II like lung epithelial cell line like, were done. From cytotoxicity view microparticles contain CIP without excipient and CIP with LEU or combinations of excipients did not change the impedance of A549 lung epithelial cell monolayers in the range of 1-300 µM concentrations, indicating no cellular toxicity As a result, microparticles did not change the impedance of lung epithelial cell and did not show any cellular toxicity. For the permeability test lung epithelial cells were co-cultured with endothelial cells for ten days. From permeability perspective formulations have same permeability value compared to CIP formulations.

6. NOVELTY AND PRACTICAL ASPECTS

This study has created the ability of development of DPI and spray-drying techniques to produce microparticles containing CIP for pulmonary drug delivery. The development of DPI of antibiotics in carrier- free system is a novelty for local treatment of respiratory tract infection.

- In this current work the application of an up-to-date and regulatory-based pharmaceutical quality management method demonstrated as a new development concept in the process of formulating DPI. This formulation produced according to the QbD methodology and Risk Assessment thinking. This innovative formulation technology and product appear to have a great potential in pulmonary drug delivery. Subsequent to no examples of QbD and RA based CIP containing DPI system formulation have been described so far.
- Co-spray-drying of CIP from an aqueous solution as innovative technology was used to prepare the novelty-type of microparticles. The advanced technology was prepared the formulation of CIP in one-step and fast process. The final microparticles which developed in green technology ensures the respirable particle size range (3-5 µm), with spherical morphology. The microparticles displayed an enhanced aerosol performance with fine particle fraction up to 80%. This high ability to aerosolization of particles is uniqueness in the DPI development.
- The formulated microparticles as innovative product tested for the stability in stress and accelerated test in long term (6 months). Since the microparticles in the dry powder system are amorphous and do not contain any stabilizer, the results of this test are very important. The stable product which may be considered suitable for scaled-up processes and pulmonary application.
- The formulated DPI illustrate a novel possibility in treatment of respiratory tract infection and the innovative technology and product present to be of great potential in pulmonary drug delivery systems.

References

Adi H, Young P. Controlled release antibiotics for dry powder lung delivery. Journal of Pharmaceutical Sciences 2010; 36: 119–126. https://doi.org/10.3109/03639040903099769

Ambrus R, Pomázi A, Réti-Nagy K, Fenyvesi F, Vecsernyés M, Szabó-Révész P. Cytotoxicity testing of carrier-based microcomposites for DPI application. Pharmazie 2011; 66: 549–550. https://doi.org/ 10.1691/ph .2011.0378

Anderson J. History of aerosol therapy liquid nebulization to MDIs to DPIs. Conference Proceedings 2005; annotated (2)

Andrade F, Rafael D, Videira M, Ferreira D, Sosnik A, Sarmento B. Nanotechnology and pulmonary delivery to overcome resistance in infectious diseases. Advanced Drug Delivery 2013; 65: 1816–1827 https://doi.org/10.1016/j.addr.2013.07.020

Antoniu S.A, Cojocaru I, 2012. Inhaled colistin for lower respiratory tract infections. Expert Opinion. Drug Delivery 2012; 9: 333–42 https://doi.org/10.1517/17425247.2012.660480

Arafa M, Abdel-Wahab H, El-Shafeey F, Badary A, Hamada A. Anti-fibrotic effect of meloxicam in a murine lung fibrosis model. European Journal of Pharmacology 2007; 564: 181–189 https://doi.org/10.1016/j.ejphar.2007.02.065

Bajaj S, Singla D, Sakhuja N. 2012. Stability Testing of Pharmaceutical Products. Journal of Applied Pharmaceutical Science; 2: 129–138 DOI: 10.7324/JAPS.2012.2322

Batchelor K. 1976. Brownian diffusion of particles with hydrodynamic interaction. Journal of Fluid Mechanics 1976; 74: 1–29 https://doi.org/10.1017/S0022112076001663

Belotti S, Rossi A, Colombo P, Bettini R, Rekkas D, Politis S, Colombo G, Balducci A.G, Buttini F.Spray dried amikacin powder for inhalation in cystic fibrosis patients: A quality by design approach for product construction. International Journal of Pharmaceutics 2014; 471: 507–515 https://doi.org/ 10.1016/j.ijpharm .2014.05.055

Blau H, Mussaffi H, Mei Zahav M, Prais D, Livne M, Czitron B.M, Cohen H. 2007. Microbial contamination of nebulizers in the home treatment of cystic fibrosis. Child: Care, Health and Development; 33: 491–495 https://doi.org/10.1111/j.1365-2214.2006.00669.x

Bolon M.K. 2011. The Newer Fluoroquinolones. The Medical clinics of North America 2011; 95:793–817 https://doi.org/10.1016/j.mcna.2011.03.006

Boraey M.A, Hoe S, Sharif H, Miller D.P, Lechuga-Ballesteros D, Vehring R. Improvement of the

dispersibility of spray-dried budesonide powders using leucine in an ethanol-water cosolvent system. Powder Technology 2013; 236: 171–178 https://doi.org/10.1016/j.powtec.2012.02.047

Camelo A, Dunmore R, Sleeman M.A, Clarke D.L. The epithelium in idiopathic pulmonary fibrosis: Breaking the barrier. Frontiers in Pharmacology 2014; https://doi.org/10.3389/fphar.2013.00173

Carvalho T.C, Peters J.I, Williams R.O. Influence of particle size on regional lung deposition – What evidence is there? International journal of Pharmaceutics 2011; 406: 1–10 https://doi.org /10.1016/j .ijpharm .2010.12.040

Charoo N.A, Shamsher A, Zidan A.S, Rahman. Quality by design approach for formulation development : A case study of dispersible tablets. International journal of pharmaceutics 2012; 423: 167–178 https://doi.org/10.1016/j.ijpharm.2011.12.024

Chatterjee S. Role of Models in the Quality by Design (QbD) Paradigm: Regulatory Perspective 2011. American Association of pharmaceutical scientists.

Cipolla D, Chan H-K. Inhaled antibiotics to treat lung infection. Pharmaceutical patent analyst 2013; 2: 647–663. https://doi.org/10.4155/PPA.13.47

Davis P.B. Cystic fibrosis since 1938. American journal of American Journal of Respiratory and Critical Care Medicine 2006. https://doi.org/10.1164/rccm.200505-840OE

De Barros M, Perciano P.G, Dos Santos M.H, De Oliveira L, Costa É.D.M, Moreira M.A.S. Antibacterial activity of 7-epiclusianone and its novel copper metal complex on streptococcus spp. isolated from bovine mastitis and their cytotoxicity in MAC-T cells. Molecules 2017; 22 : 823 https://doi.org/10.3390/molecules22050823

de Boer A.H, Hagedoorn P, Hoppentocht M, Buttini F, Grasmeijer F, Frijlink H.W.2017. Dry powder inhalation: past, present and future. Expert Opinion in Drug Delivery 2017; 14 https://doi.org /10.1080 /17425247 .2016.1224846

Desal K.R. Green Chemistry : New Methods for Organic Synthesis and Applications. Green Chem. Eng 1981; 1-20

Deli M.A. Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. Biochimica et Biophysica Acta - Biomembranes 2009; 1788: 892–910. https://doi.org/10.1016/j.bbamem.2008.09.016

EMA/CHMP, ICH guideline Q9 on quality risk management. 2014 http://www.ema.europa.eu/docs/en _GB/document_library/Scientific_guideline/2009/09/WC500002873.pdf. Accessed May 5, 2016.

EMA, CHMP, Guideline on the Pharmaceutical Quality of Inhalation and Nasal Products. 2006 [http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC50000

3568.pdf. Accessed May 5, 2016.]

EMEA/CHMP, ICH Topic Q 8 (R2) Pharmaceutical Development, Step 5: Note For Guidance On PharmaceuticalDevelopment.2009[http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500059258.pdf. Accessed May 5, 2016.]

EMA, CPMP, Note for guidance for dry powder inhalers, 1998. European Medicines Agency, n.d. Quality by design [WWW Document]. Hum. Regul.

FDA, Guidance for Industry-Clinical Development Programs for MDI and DPI Drug Products, 1994 http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformationGuidances/UCM071955.pdf. Accessed May 5, 2016.

FDA, Guidance for Industry Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) Drug Products, Chemistry, Manufacturing, and Controls Documentation, n.d. . 1998.

Fujii H. Decomposition analysis of green chemical technology inventions from 1971 to 2010 in Japan. Journal of Cleaner Production 2016; 112: 4835–4843. https://doi.org/10.1016/j.jclepro 2015.07.123

Geller D.E, Konstan M.W, Smith J, Moonberg S.B, Conrad C. Novel tobramycin inhalation powder in cystic fibrosis subjects: Pharmacokinetics and safety. Pediatric Pulmonology 2007; 42: 307–313 https://doi.org/10.1002/ppul.20594

Gelperina S, Kisich K, Iseman M.D, Heifets L. The Potential Advantages of Nanoparticle Drug Delivery Systems in Chemotherapy of Tuberculosis. American Journal of Respiratory and Critical Care Medicine 2005; 172: 1487–1490. https://doi.org/10.1164/rccm.200504-613PP

Greally P, Whitaker P, Peckham D. Challenges with current inhaled treatments for chronic Pseudomonas aeruginosa infection in patients with cystic fibrosis. Current Medical Research and Opinion 2012; 28: 1059–1067 https://doi.org/10.1185/03007995.2012.674500

Grgic B, Finlay W.H, Heenan A.F. 2004. Regional aerosol deposition and flow measurements in an idealized mouth and throat. Journal of Aerosol Science 2004; 35: 21–32 https://doi.org/ 10.1016/S0021-8502(03)00387-2

Hamishehkar H, Rahimpour Y, Javadzadeh Y. The role of carrier in dry powder inhaler. In Techology Open Access Publication 2012; 39–66. https://doi.org/10.5772/51209

Healy A.M, Amaro M.I, Paluch K.J, Tajber L. Dry powders for oral inhalation free of lactose carrier particles. Advanced Drug Delivery 2014; 75: 32–52. https://doi.org/10.1016/j.addr.2014.04.005

Heyder J, Gebhart J, Rudolf G, Schiller C.F, Stahlhofen W. Deposition of particles in the human respiratory tract in the size range 0.005-15 μm. Journal of . Aerosol Science1986; 17: 811–825

https://doi.org/10.1016/0021-8502(86)90035-2

.Hickey A.J, Pharmaceutical inhalation Aerosol technology 2003; second edition:215-253

Hofmann W. Modelling inhaled particle deposition in the human lung-A review. Journal of Aerosol Science 2011; 42: 693–724 https://doi.org/10.1016/j.jaerosci.2011.05.007

Hoiby N. Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. BMC Medicine 2011; 9–32. https://doi.org/10.1186/1741-7015-9-32

Hoppentocht M, Hagedoorn P, Frijlink H.W, de Boer A.H. Technological and practical challenges of dry powder inhalers and formulations. Advanced Drug Delivery 2014; 75: 18–31. https://doi.org/ 10.1016/j .addr.2014.04.004

Horváth T, Bartos C, Bocsik A, Kiss L, Veszelka S, Deli M, Újhelyi G, Szabó-Révész P, Ambrus R. Cytotoxicity of Different Excipients on RPMI 2650 Human Nasal Epithelial Cells. Molecules 2016; 21: 658-663 https://doi.org/10.3390/molecules21050658

Huang J, Kaul G, Cai C, Chatlapalli R, Hernandez-Abad P, Ghosh K, Nagi A. Quality by design case study: An integrated multivariate approach to drug product and process development. International journal of pharmaceutics 2009; 382: 23-32 https://doi.org/10.1016/j.ijpharm. 2009.07.031

Heyder J, Gebhart J, Rudolf G, Schiller C.F, Stahlhofen W.S. Deposition of Particles in the Human Respiratory. Journal of Aerosol Science 1986

Islam N, Gladki E. Dry powder inhalers (DPIs)—A review of device reliability and innovation. International journal of pharmaceutics 2008; 360: 1–11 https://doi.org/10.1016/j.ijpharm. 2008.04.044

Inhalanda. Preparations for inhalation. Pharm Eur Suppl 1998; 0671: 984–989

Kakkar A. P., Manmohan Sinagh, and Arun Mendiratta, Isolation and Characterization of Ciprofloxacin-HCL Crystals, Drug Development and Industrial pharmacy. 11 (1997) 1063-1067

Karimi K, Pallagi E, Szabó-Révész P, Csoka I, Ambrus R. Development of a microparticle-based dry powder inhalation formulation of ciprofloxacin hydrochloride applying the quality by design approach: Drug Design, Development and Therapy Journal 2016;10: 3331-3343

Karimi K, Katona G, Csoka I, Ambrus R. Physicochemical stability and aerosolization performance of dry powder inhalation system containing ciprofloxacin hydrochloride: Journal of Pharmaceutical and Biomedical Analysis 2018;148: 73-79

Konstan M.W, Geller D.E, Mini P, Brockhaus F, Zhang J, Angyalosi G. Tobramycin inhalation powder for P. aeruginosa infection in cystic fibrosis: the EVOLVE trial. NIH Public Access 2011; 46: 230–

238 https://doi.org/10.1002/ppul.21356

Krátký M, Dzurková M, Janoušek J, Konečná K, Trejtnar F, Stolaříková J, VinŠová J. Sulfadiazine salicylaldehyde-based schiff bases: Synthesis, antimicrobial activity and cytotoxicity. Molecules 2017; 22: 1–15. https://doi.org/10.3390/molecules22091573

Láng P, Várkonyi E, Ulrich J, Szabó-Révész P, Aigner Z. Analysis of the polymorph changes of a drug candidate. Journal of Pharmaceutical and Biomedical Analysis 2015; 102: 229–235. https://doi.org/10.1016/j.jpba.2014.09.020

LeBel M. Ciprofloxacin: Chemistry, Mechanism of Action, Resistance, Antimicrobial Spectrum, Pharmacokinetics, Clinical Trials, and Adverse Reactions. American college of clinical Pharmacy 1988; 8: 3-30 https://doi.org/10.1002/j.1875-9114.1988.tb04058.x

Li L, Sun S, Parumasivam T, Denman J.A, Gengenbach T, Tang P, Mao S, Chan H. European Journal of Pharmaceutics and Biopharmaceutics as an excipient against moisture on in vitro aerosolization performances of highly hygroscopic spray-dried powders L -Leucine 2016. 102: 132–141. https://doi.org /10.1016/j.ejpb.2016.02.010

Littlewood K.J, Higashi K, Jansen J.P, Capkun-Niggli G, Balp M.M, Doering G, Tiddens H.A.W.M, Angyalosi G. A network meta-analysis of the efficacy of inhaled antibiotics for chronic Pseudomonas infections in cystic fibrosis. J. Cyst. Fibros 2012; 11: 419–426 https://doi.org /10.1016/j.jcf.2012.03.010

Marslin G, Siram K, Liu X, Khandelwal V.K.M, Shen X, Wang X, Franklin G, Solid lipid nanoparticles of albendazole for enhancing cellular uptake and cytotoxicity against u-87 mg glioma cell lines. Molecules 2017; 22 https://doi.org/10.3390/molecules22112040

Masadeh M.M, Alzoubi K.H, Khabour O.F, Al-Azzam S.I. Ciprofloxacin-Induced Antibacterial Activity Is Attenuated by Phosphodiesterase Inhibitors. Current Therapeutic Research 2014; 77: 14–17. https://doi.org/10.1016/j.curtheres.2014.11.001

Michael E.Aulton, K.M.G.T., 2018. Aulton's Pharmaceutics.

Miller D.A, Ellenberger D, Gil M. Spray-drying technology. Formulating poorly water soluble drugs 2016; 437-525 https://doi.org/10.1007/978-3-319-42609-9_10

Newman S.P. Aerosol deposition considerations in inhalation therapy. Chest Journal 1985; 88: 152–160

Ng M.Y, Flight W, Smith E. Pulmonary complications of cystic fibrosis. Clinical Radiology 2014; 69: 153-169 https://doi.org/10.1016/j.crad.2013.10.023

O'Sullivan B.P, Freedman S.D. Cystic fibrosis. Lancet 2009; 373: 1891–1904 https://doi.org /10.1016/

S0140-6736(09)60327-5

Pallagi E, Ambrus R, Szabó-Révész P, Csóka I. Adaptation of the quality by design concept in early pharmaceutical development of an intranasal nanosized formulation. Int. J. Pharm 2015; 491: 384-392 https://doi.org/10.1016/j.ijpharm.2015.06.018

Pallagi E, Karimi K, Ambrus R, Szabó-Révész P, Csóka I. New aspects of developing a dry powder inhalation formulation applying the quality-by-design approach. Int. J. Pharm 2016; 511: 151-160 https://doi.org/10.1016/j.ijpharm.2016.07.003

Pilcer G, De Bueger V, Traina K, Traore H, Sebti T, Vanderbist F, Amighi K. Carrier-free combination for dry powder inhalation of antibiotics in the treatment of lung infections in cystic fibrosis. Int. J. Pharm 2013; 451: 112–120 https://doi.org/10.1016/j.ijpharm.2013.04.069

Pilcer G, Rosière R, Traina K, Sebti T, Vanderbist F, Amighi K. New co-spray-dried tobramycin nanoparticles-clarithromycin inhaled powder systems for lung infection therapy in cystic fibrosis patients. Journal of pharmaceutical science 2013; 102: 1836–1846. https://doi.org/10.1002 /jps.23525

Pilcer G, Sebti T, Amighi K. Formulation and characterization of lipid-coated tobramycin particles for dry powder inhalation. Pharm. Res 2006; 23: 931–940. https://doi.org/10.1007/s11095-006-9789-4

Pitha J, Milecki J, Fales H, Pannell L, Uekama K. Hydroxypropyl-β-cyclodextrin: preparation and characterization; effects on solubility of drugs. Int. J. Pharm 1986; 29: 73-82 https://doi.org/ 10.1016/0378-5173(86)90201-2

Pohl C, Hermanns M.I, Uboldi C, Bock M, Fuchs S, Dei-Anang J, Mayer E, Kehe K, Kummer W, Kirkpatrick C.J. Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. Eur. J. Pharm. Biopharm 2009; 72: 339–349. https://doi.org/10.1016/ j.ejpb.2008.07.012

Pomázi A, Ambrus R, Szabó-Révész P. Physicochemical stability and aerosolization performance of mannitol-based microcomposites. J. Drug Deliv. Sci. Technol 2014; 24: 397–403. https://doi.org/10.1016/S1773-2247(14)50080-9

Pomázi A, Buttini F, Ambrus R, Colombo P, Szabó-Révész P. Effect of polymers for aerolization properties of mannitol-based microcomposites containing meloxicam. European Polymer Journal 2013; 49: 2518-2527 https://doi.org/10.1016/j.eurpolymj.2013.03.017

Prota L., Santoro A., Bifulco M., Aquino R. P., Mencherini T., Russo P. Leucine enhances aerosol performance of Naringin dry powder and its activity on cystic fibrosis airway epithelial cells. Int. J.

Pharm., 412 (2011) 8–19.

Quality by Design for ANDAs : An Example for Immediate-Release Dosage Forms. 2012; 1–107.

Quon B.S, Goss C.H, Ramsey B.W. Inhaled antibiotics for lower airway infections. Annals of the American Thoracic Society 2014; 11: 425–434. https://doi.org/10.1513/AnnalsATS.201311-395FR

Rabel S.R, Jona J.A, Maurin M.B. Applications of modulated differential scanning calorimetry in preformulation studies. Journal of pharmaceutical and biomedical analysis 1999; 21: 339–345

Raula J, Thielmann F, Naderi M, Lehto V.P, Kauppinen E.I. Investigations on particle surface characteristics vs. dispersion behaviour of l-leucine coated carrier-free inhalable powders. Int. J. Pharm 2010; 385: 79–85 https://doi.org/10.1016/j.ijpharm.2009.10.036

Sanders M. Inhalation therapy: an historical review. Prim. Care Respir. J 2007; 16: 71–81. https://doi.org/10.3132/pcrj.2007.00017

Satoh K. Poly(vinyl alcohol) (PVA). Encyclopedia of Polymeric Nanomaterials 2014; 13-19 https://doi.org/10.1007/978-3-642-36199-9_246-1

Schumacher J, Leiner S. 2012. A critical evaluation of the revised and new USP Chapters for Aerosols. Pharm Forum 2011; 37: 1–6

Singh B, Dahiya M, Saharan V, Ahuja N. Optimizing drug delivery systems using systematic "design of experiments." Part II: Retrospect and prospects. Critical Reviews in Therapeutic Drug Carrier Systems 2005; 22: 215-294 https://doi.org/10.1615/CritRevTherDrugCarrierSyst.v22.i3.10

Sosnik A, Seremeta K.P. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv. Colloid Interface Sci 2015; 223: 40–54 https://doi.org/10.1016/j.cis.2015.05.003

Sousa A.M, Pereira M.O. Pseudomonas aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs-A Review. Pathog. (Basel, Switzerland) 2014; 3: 680–703 https://doi.org/ 10.3390/pathogens3030680

Sung J.C, Pulliam B.L, Edwards D.A. Nanoparticles for drug delivery to the lungs. Trends Biotechnol 2007; 25: 563–570. https://doi.org/10.1016/j.tibtech.2007.09.005

Suresh A.K, Pelletier D.A, Wang W, Morrell-Falvey J.L, Gu B, Doktycz M.J. Cytotoxicity induced by engineered silver nanocrystallites is dependent on surface coatings and cell types. Langmuir 2012; 28: 2727-2735 https://doi.org/10.1021/la2042058

Telko M.J, Hickey A.J. Dry powder inhaler formulation. Respiratory Care 2005; 50: 1209-27

To T, Cpmp T.H.E, Of A, Revision T.H.E, The B.Y, For D, Into C. Guideline on Stability Testing :

Stability Testing of Existing Active Substances and Related Finished Products. SubStance 2004; 1–18

Tomba E, Facco P, Bezzo F, Barolo M. Latent variable modeling to assist the implementation of Quality-by-Design paradigms in pharmaceutical development and manufacturing: A review. Int. J. Pharm 2013; 457: 283-297 https://doi.org/10.1016/j.ijpharm.2013.08.074

U.S. Department of Health and Human Services, 1998. Metered Dose Inhaler (MDI) and Dry Powder Inhaler (DPI) Drug Products: Chemistry, Manufacturing, and Controls Documentation 1–66.

Vehring R. Pharmaceutical particle engineering via spray drying. Pharm. Res 2008; 25: 999-1022 https://doi.org/10.1007/s11095-007-9475-1

Weers J.G, Miller D.P. Formulation Design of Dry Powders for Inhalation. J. Pharm. Sci 2015; 104: 3259–3288 https://doi.org/10.1002/jps.24574

Westerman E.M, De Boer A.H, Le Brun P.P.H, Touw D.J, Roldaan A.C, Frijlink H.W, Heijerman H.G.M. Dry powder inhalation of colistin in cystic fibrosis patients: A single dose pilot study. J. Cyst. Fibros 2007; 6: 284–292 https://doi.org/10.1016/j.jcf.2006.10.010

Wilson R, Welte T, Polverino E, De Soyza A, Greville H, O'Donnell A, Alder J, Reimnitz P, Hampel B. Ciprofloxacin dry powder for inhalation in non-cystic fibrosis bronchiectasis: A phase II randomised study. Eur. Respir. J 2013; 41: 1107–1115 https://doi.org/10.1183/09031936. 00071312

World Health Organization. The Global Burden of Disease: 2008 update, 146. https://doi.org/ 10.1038/npp.2011.85

Wu L, Miao X, Shan Z, Huang Y, Li L, Pan X, Yao Q, Li G, Wu C. Studies on the spray dried lactose as carrier for dry powder inhalation. Asian J. Pharm. Sci 2014; 9: 336–341. https://doi.org/10.1016/ j.ajps.2014.07.006

Yang Y, Bajaj N, Xu P, Ohn K, Tsifansky M.D, Yeo Y. Development of highly porous large PLGA microparticles for pulmonary drug delivery. Biomaterials 2009; 30: 1947–1953 https://doi.org/ 10.1016/j.biomaterials.2008.12.044

Yu H, Teo J, Chew J.W, Hadinoto K. Dry powder inhaler formulation of high-payload antibiotic nanoparticle complex intended for bronchiectasis therapy: Spray drying versus spray freeze drying preparation. Int. J. Pharm 2016; 499: 38–46. https://doi.org/10.1016/j.ijpharm. 2015.12.072

Yu L.X. Pharmaceutical quality by design: Product and process development, understanding and control. Pharm. Res 2008; 25: 781-791 https://doi.org/10.1007/s11095-007-9511-1

Yu L.X, Amidon G, Khan M.A, Hoag S.W, Polli J, Raju G.K, Woodcock J. 2014. Understanding

pharmaceutical quality by design. AAPS J 2014;. 16: 771–83 https://doi.org/10.1208/s12248-014-9598-3

Zhao H, Le Y, Liu H, Hu T, Shen Z, Yun J, Chen J.F. Preparation of microsized spherical aggregates of ultrafine ciprofloxacin particles for dry powder inhalation (DPI). Powder Technol 2009; 194: 81–86. https://doi.org/10.1016/j.powtec.2009.03.031

Zidan A.S, Sammour O.A, Hammad M.A, Megrab N.A, Habib M.J, Khan M.A. Quality by design: Understanding the formulation variables of a cyclosporine A self-nanoemulsified drug delivery systems by Box-Behnken design and desirability function. Int. J. Pharm 2007; 332: 55-63 https://doi.org/ 10.1016/j.ijpharm.2006.09.060