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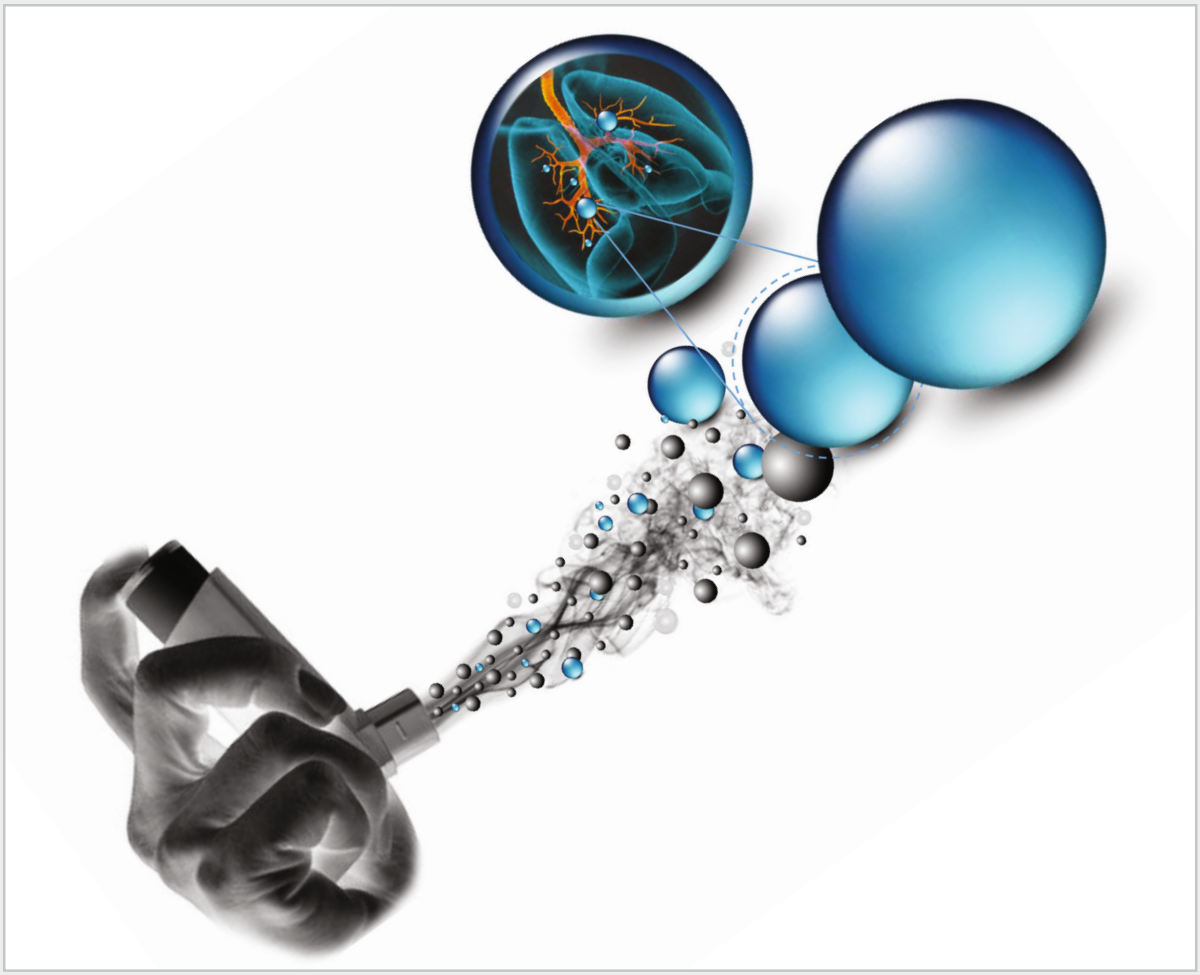
Acta Pharmaceutica Hungarica

pulmonary drug delivery



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Pulmonary drug delivery: Role of antibiotic formulations for treatment of respiratory tract infections

ILDIKÓ CSÓKA, KEYHANEH KARIMI, MAHWASH MUKHTAR, RITA AMBRUS*

Institute of Pharmaceutical Technology and Regulatory Affairs, Faculty of Pharmacy, Interdisciplinary Excellence Centre, University of Szeged

Corresponding author: Rita Ambrus, Email: arita@pharm.u-szeged.hu

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Abstract

Respiratory infections cause an extensive health problem in the world. The common treatment for respiratory infections is the administration of antibiotics orally or parenterally in a high dose. Unfortunately, these therapies of high-dose antimicrobials have many disadvantages, such as severe side effects. Consequently, the development of an inhaled formulation provides the delivery of the therapeutic dose of the drug to the organ of interest without overt systemic effects. Novel technological advances have led to the development of inhaled antibiotics. Recent particle engineering techniques for dry powder inhalers (DPI) or mesh nebulizers have higher aerosolization efficiencies and promote the delivery of high-dose antibiotics to the lungs. However, advanced formulation strategies are in high demand for the development of new formulations for more types of antibiotics. Despite all the current research, patient compliance with pulmonary dosage forms remains to be very low because of the inappropriate administration techniques. Hence, this review focuses on three key aspects of the pulmonary dosage forms of antibiotics; the marketed products, the formulation approaches under research and innovative formulation strategies for achieving drug delivery through the respiratory tract.

Key words: *antibiotic dosage form, Inhaled formulation, dry powder inhalation, nebulization, particle design, antibiotic combination, ishikawa diagram*

1. Introduction

Inhaled therapy for medicinal purposes was used at least 4,000 years ago, but using antibiotics in a pulmonary dosage form takes back to 1948, when Abbot Laboratories developed the Aerohalor for the inhalation of Penicillin G powder [1]. However, large-scale therapeutic advancement dates back to 1997, when tobramycin for inhalation was approved by the U.S. Food and Drug Administration (FDA) for use in patients with cystic fibrosis [2]. Respiratory tract infections affect people in all ages and are very common [3-5]. Globally, infections of the lower respiratory tract are among the top three major causes of morbidity and every year, these can be responsible for approximately 3.5 million deaths in the world [6]. The most common treatment for respiratory infections involves the oral or parenteral administration of high doses of single or combined antibiotics, which can show undesirable side effects because of high systemic bioavailability [7, 8]. The ability to deliver therapeutic agents to the site of action may allow efficient treatments of infectious diseases of the respiratory tract and has many advantages over other routes [9].

The large surface area of the lungs is supplied by the excessive blood capillary network, which plays a role in the rapid absorption of the drug in the lungs. So the absorbed drug can directly reach the blood circulation, thus evading first pass metabolism through this non-invasive drug delivery system [10, 11].

Therefore, the delivery of even low concentrations of antibiotics to the lungs at the site of infection leads to much higher concentrations of antibiotics in the lungs, while reducing systemic exposure and the risk of toxicity, and yielding therapeutic effects with smaller drug doses than the oral or parenteral route [12, 13].

One example is when an aminoglycoside antibiotic amikacin is given by IV administration, the drug concentration in the serum is three times higher than in the bronchial tissues, however, when amikacin is given by inhalation, drug concentration is 1000 times higher in the bronchial tis-

sues than in the serum [14]. The other advantage of using a pulmonary dosage form of antibiotics in the treatment of chronic infections is that it is not associated with pain and it increases patient comfort and compliance, causing rational treatment outcome. Therefore, it enhances the quality of life, shortens the hospitalization period and significantly decreases morbidity and mortality [15-18].

Currently, there are no drugs available that can stop the progression of Chronic Obstructive Pulmonary Disease (COPD), but inhaled therapies have proved fruitful by preventing progression in many trials. Previously, many oral therapies were available for the treatment of idiopathic pulmonary hypertension, however, new interventions are needed to increase patient compliance, which may affect disease progression. There has also been an increase in the development of aerosolized liposomal formulations for the treatment of pulmonary neoplasia and 9-nitro-20(S)-camptothecin liposomal therapy has already been in clinical trials [19].

In spite of the great advantages of pulmonary dosage forms of antibiotics for the treatment of respiratory infection, there are a few disadvantages for this route, which cause limitations when using antibiotics in a pulmonary dosage form [20]. Metabolic enzymes found in the lungs metabolize the antibiotics, however, the pathways and metabolic activities are different from degradation observed in the gastrointestinal tract [21]. Antibiotics can be cleared by the activity of alveolar macrophages found in the pulmonary alveoli, and the activity of these macrophages is relatively high since they are located at one of the extensive borders between the body and the outside environment [22, 23]. On the other hand, the inhalation of antibiotics may cause severe local irritation, wheezing, bronchospasm and coughing in patients [24].

In consideration of all these advantages and disadvantages, the development of inhaled antibiotics to treat lung infection is a largely active field, with four approved products in the USA and others in the late stages of clinical progress [13].

In this review, we have discussed the pulmonary dosage forms of antibiotics for the treatment of respiratory infections. Then particular inhaled formulations have been reviewed, highlighting fields where further research is required, e.g., particle engineering and particle preparation, and innovative formulations of pulmonary dosage forms. Then different methods of liposome preparation of antibiotics have been reviewed, and finally some of the formulations which contain a combination of antibiotics have been studied.

2. Inhaled antibiotic formulations in the market and in the development phase

2.1. Approved antibiotic formulations and their delivery routes

The comparison of the general treatment guideline of respiratory tract infections and literature data about the pulmonary dosage form of antibiotics shows that these two lines are not parallel. The data show most research and investigations of pulmonary dosage forms of antibiotics focus on the treatment of cystic fibrosis rather than generally on the treatment of respiratory tract infection [25-27]. Cystic Fibrosis (CF) is an inherited disease caused by different mutations of the transmembrane conductance regulatory gene, and consequently respiratory failure follows after the chronic inflammation of the respiratory tract [28-30]. Currently, CF is the particular pulmonary infectious disease in which inhaled antibiotics have received FDA and European Medicines Agency (EMA) approval [24]. Future inhaled antibiotic trials have to focus on pulmonary diseases other than CF with large-scale manufacturing of marketed products for treating the variety of pulmonary infections. Hence, the pulmonary dosage form of antibiotics can be used for the treatment of upper respiratory tract infections such as pharyngitis, tonsillitis, laryngitis, tracheitis, or lower respiratory tract infections like acute bronchitis and pneumonia often following after common cold and influenza [31-33]. Therefore, there will be a possibility for eradicating a broad spectrum of Gram-positive and Gram-negative bacteria from the respiratory system. The antibiotics discussed in this review have been a subject of research and investigation over the years. Nowadays they are under clinical investigation or marketing. Table I illustrates the groups of antibiotics in different dosage forms that are present in the market. So based on the data of table 1, we can assume that many different oral and parenteral dosage forms of antibacterial agents have been formulated; however, as Figure 1 shows, only 0.01% of antibiotic formulations exist in pulmonary dosage form and 0.04% of the total group of antibiotics involves inhaled formulations. Figure 2 shows the ratio of different products in the USA and the UK.

Table I *Summary of different key formulations of antibiotics in the USA and the UK/Europe. (Compiled from EMA's website:* www.ema.europa.eu*, FDA's database:* www.fda.gov/home*, National Institute of Pharmacy and food website:* www.ogyei.gov.hu*)*

Group of Penicillins	**USA**	**UK/Europe**
Amoxicillin	Capsule, Chewable tablet, Drops, Extended-release tablet, Tablet for suspension, Suspension	Oral hard Capsule, Dipersible tablet, Oral suspension, Powder for oral suspension, Powder for solution for injection and infusion
Ampicillin	Injection, Solution, Suspension, Powder for solution for injection, Capsules	Oral suspension, Powder for solution for injection, Powder for oral suspension, Capsules
Dicloxacillin	Capsule, Oral suspension	
Carbenicillin indanyl	Capsule	Capsule
Nafcillin	Injection, Infusion	
Oxacillin	Powder for injection, Infusion solution, Tablet	Capsule, Powder for solution for injection
Penicillin G	Injection, Infusion, Tablet	Injection, Infusion, Powder for injection, Tablet
Penicillin V	Oral solution, Tablet	Oral solution, Tablet
Piperacillin	Injection, Infusion	Injection, Infusion
Ticarcillin	Infusion	Infusion
Group of Cephalosporins	**USA**	**UK/Europe**
Cefaclor	Capsule, Film coated tablet, Paedetric drops	Capsule, Suspension, Powder for suspension
Cefadroxil	Capsule, Suspension, Tablet	Capsule, Granule for oral suspension
Cefazolin	Injection	Powder for injection/ infusion
Cefdinir	Suspension, Capsule	
Cefepime	Injection	
Cefixime	Tablets, Suspension	Granules for oral suspension, Film coated tablets, Powder for oral suspension
Cefotaxime	Injection	Powder for solution, Powder for injection or infusion
Cefotetan	Injection	
Cefoxitin	Infusion	
Cefprozil	Suspension, Tablets	
Ceftazidime	Injection	Powder for solution, Powder for injection or infusion
Ceftibuten	Suspension	
Ceftizoxime	Injection	Injection
Ceftaroline	Intravenous powder for injection	Intravenous powder for injection
Ceftriaxone	Injection	Powder for solution, Powder for injection or infusion
Cefuroxime	Injection, Infusion, Suspension, Tablet	Film-coated tablet, Suspension, Granules for suspension, Tablet, Powder for injection or infusion
Cephalexin	Capsule, Oral suspension, Tablet	Capsule, Oral suspension, Tablet
Group of Carbapenems	**USA**	**UK/Europe**
Doripenem	Injection	
Ertapenem	Injection	Powder for concentrate for solution for infusion
Imipenem	For injection (combination with Cilastatin)	Powder for solution for infusion (combination with Cilastatin)
Meropenem	Powder for injection/infusion	Powder for solution for injection/infusion
Group of Monobactams	**USA**	**UK/Europe**
Aztreonam	Inhalation, Injection, Infusion	Injection
Group of Tetracyclines	**USA**	**UK/Europe**
Demeclocycline	Capsule, Tablet	Capsule, Tablet
Doxycycline	Capsule, Injection, Delayed release tablet, Subgingival controlled release gel	Capsule, Tablet

Minocycline	Extended release capsule, Extended release tablet, Injection, Sublingual, Oral microspheres	Film-coated tablet, Tablet, Capsule
Tetracycline	Tablet, Capsule, Suspension, Topical, Eye ointment	Capsule, Tablet, Eye ointment
Group of Glycylcyclines	**USA**	**UK/Europe**
Tigecycline	Injection	Injection
Group of Aminoglycosides	**USA**	**UK/Europe**
Amikacin	Injection	Injection, Solution for infusion
Gentamicin	Cream, Drops, Injection, Ointment, Solution, Topical	Drops, Infusion, Injection, Solution for injection
Neomycin	Cream, Drops, Injection, Ointment, Solution	Tablet, Drops
Streptomycin	Injection	Injection
Tobramycin	Eye drops, Injection, Inhalation solution, Ointment, Powder, Ophthalmic Solution	Injection, Infusion, Ointment, Eye drops
Group of Macrolides	**USA**	**UK/Europe**
Azithromycin	Injection, Suspension, Tablet, Capsule, Ophthamlic solution, Powder for suspension	Capsule, Film coated tablet, Injection, Suspension, Powder for oral suspension, Powder for solution for injection
Clarithromycin	Extended release tablet, Suspension	Film coated tablet, Granules for Oral suspension, Powder for solution for infusion, Tablet
Erythromycin	Capsule, Delayed release tablet, Delayed release Capsule, Drops, Gel, Infusion, Ointment, Oral suspension, Solution, Infusion, Tablet, Topial pad	Film coated tablet, Gastro-resistant tablet, Granules for oral suspension, Oral suspension, Powder for solution for infusion, Sugar free Oral suspension, Tablet
Telithromycin	Tablet	Film tablet
Group of Fluoroquinolones	**USA**	**UK/Europe**
Nalidixic acid	Suspension, Tablet	Tablet
Ciprofloxacin	Drop, Extended release tablet, injection, Infusion, Ointment, Suspension, Solution, Tablet	Drop, Film coated tablet, Granules for oral suspension, Solution for infusion, Tablet
Norfloxacin	Drop, Suspension, Tablet	Tablet
Ofloxacin	Drop, Solution, Tablet	Tablet
Levofloxacin	Drop, Infusion, Injection concentrate, Oral solution, Solution, Tablet	Film-coated Tablet, Solution for infusion, Tablet
Moxifloxacin	Eye drops, Injection, Tablet	Film- coated tablet, Solution for infusion, Tablet, Eye drops, injection
Group of inhibitors of folate synthesis	**USA**	**UK/Europe**
Mafenide	Cream, Topical Solution	Cream, Topical solution
Silver sulfadiazine	Cream	Cream
Sulfasalazine	Delayed release tablet, Oral suspension, Rectal suspension	Enteric coated tablet, Gastro resistant tablet, Oral suspension, Tablet
Sulfisoxazole	Suspension (combination with erythromycin)	
Groups of Inhibitors of folate reduction	**USA**	**UK/Europe**
Pyrimethamine	Tablet (combination with sulfadoxine)	Tablet (combination with sulfadoxine)
Trimethoprim	Intravenous, Suspension	Suspension, Tablet
Others	**USA**	**UK/Europe**
Chloramphenicol	Capsule, Injection, Infusion	Capsule, Drops, Ointment
Clindamycin	Cream, Foam, Gel, Granules, Injection, Intravenous, Lotion, Solution, Suppository, Suspension, Swab	Capsule, Cream, Hard capsule, Solution for injection, Solution for infusion
Linezolid	Injection, Intravenous, Suspension, Tablet	Film coated tablet, Granules for oral suspension, Solution for infusion
Quinupristin / Dalfopristin	Powder for injection	

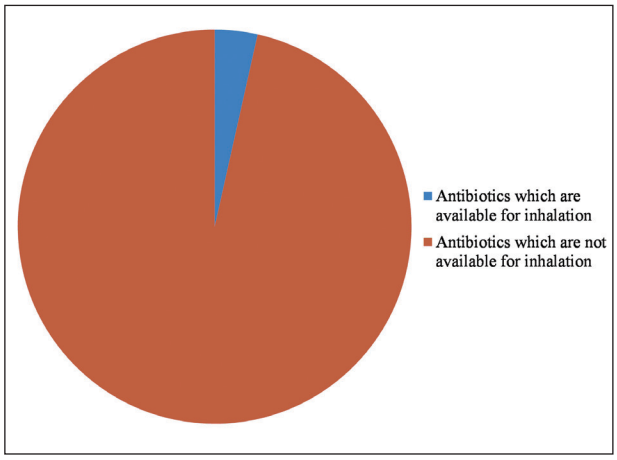

Figure 1 *Ratio of the inhaled antibiotic Vs non-inhaled antibiotic formulation*

2.2. Approved inhaled antibiotic products

2.2.1. Monobactams (Aztreonam)

β-lactam compounds are the first antibiotics to be discovered and widely used in many treatments. In this group, monobactams were developed with enhanced effect against aerobic Gram-negative bacteria. They are inactive against Gram-positive bacteria or anaerobic bacteria. They disrupt the bacterial cell wall [34]. The most common monobactam antibiotic is aztreonam [35]. Since absolute bioavailability is very low (about 1%) after oral administration, it is necessary for aztreonam to be administered intravenously or intramuscularly [36]. This drug is very safe for treating patients who are allergic to penicillins and cephalosporins [37]. Cayston®, aztreonam for inhalation solution, has been approved by FDA and EMA [38].

2.2.2. Fluoroquinolones (Ciprofloxacin, Levofloxacin)

Fluoroquinolones are potent antibacterial agents which target two enzymes, DNA gyrase and DNA topoisomerase IV [39]. Fluoroquinolones are rather well-tolerated and safe antibiotics [40]. Ciprofloxacin is the most potent fluoroquinolone for the treatment of pseudomonal infections associated with CF [41]. A liposomal ciprofloxacin formulation for inhalation is currently in clinical trials for the treatment of respiratory diseases. Dry powder formulations of ciprofloxacin are in the advanced development stage [42]. Levofloxacin is an isomer of ofloxacin, which can be utilized in a wide range of infections due to its broad spectrum of activity [43]. Nebulized levofloxacin solution, Quinsair 240 mg, is now in market [44, 45].

2.2.3. Aminoglycosides (Amikacin, Tobramycin)

Aminoglycosides are essential antibiotics in the treatment of severe and lethal infections [46]. Aminoglycosides exhibit bactericidal activity by inhibiting protein synthesis as they bind to the 30S ribosomal subunit prior to ribosome formation, therefore causing the misreading of mRNA and leaving the bacterium unable to synthesize proteins necessary for bacterium growth [47]. Liposomal amikacin suspension (Arikayce) for inhalation has been approved by FDA for the treatment of respiratory diseases [48]. Tobramycin solution (TOBI Novartis) has been approved in the USA and Europe. Dry powder inhalation tobramycin (TOBI podhaler) has been approved in the USA and Europe [49]. Tobramycin exhibits irreversible ototoxicity or nephrotoxicity as side effects, however, when administered in a pulmo-

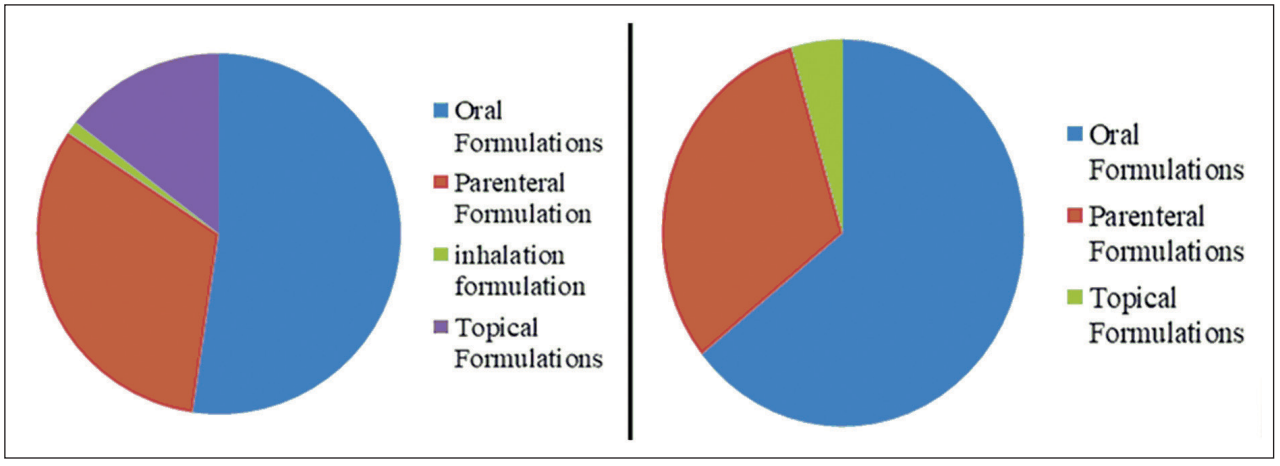

Figure 2 *Ratio of different formulations in USA (left) and UK (right)*

nary dosage form, it does not display these systemic side effects, and it is an affirming study showing that systemic toxicity can be minimized via pulmonary dosage forms [50, 51].

2.2.4. Colistin

Colistin belongs to the polypeptide antibiotics known as polymyxins. Colistin is effective against most Gram-negative bacteria. Colistin can be given intravenously and resistance to colistin is rare [52]. Colistin is polycationic and has both hydrophilic and lipophilic moieties [53]. Colistimethate sodium solution (Colomycin) has been approved in some European countries. Colistin methanesulfonate (Colobreathe) dry powder formulations have been approved in the USA and European countries. Zhou et al. reports a study which reveals that colistin in pulmonary dosage forms rarely results in systemic side effects [54-56].

2.2.5. Vancomycin

Vancomycin is a tricyclic glycopeptide antibiotic, a large hydrophilic molecule that poorly crosses the gastrointestinal mucosa. Vancomycin is effective against Gram-positive bacteria [57] and it has become extremely useful because of its effectiveness against drug-resistant organisms [58]. Dry powder of vancomycin hydrochloride (AeroVanc) for inhalation has not been marketed yet but phase I clinical study reported excellent tolerability of this antibiotic in volunteers. So dry powder formulations of vancomycin are in the upgrading development stage [59].

3. Frequently used antibiotic formulation techniques

There are numerous methods to produce pulmonary drug delivery systems; however, here we tried to focus on methods that are mostly used for pulmonary dosage forms of antibiotics.

3.1. Nebulization

Nebulized antibiotics were used for the treatment of respiratory infection in the 1950s [60]. The nebulization of antibiotics is a method for delivering therapeutic agents in a liquid form (solution or suspension) into the lungs by using nebulizing devices. Droplets with a diameter of approximately 1-5 μm are used for inhalation. This fraction can deposit in the large and small airways and the alveoli. Droplets larger than 5 μm deposit in the upper airways and droplets smaller than 1 μm are gradually exhaled again or may get into the systemic absorption [61, 62]. As a routine rule, nebulizers are suggested if the antibiotic cannot be administered using other devices [63]. Nebulizers are usually used for patients who are critically ill or children not able to use handheld devices due to the smaller geometry of the respiratory tract as well as the lower inhalation flow rates [64]. Nebulizers are also applied for any antibiotic available only in liquid form and not stable in any other form [65]. In the past, intravenous formulations were used to deliver antibiotics with different nebulizers for the treatment of serious respiratory infections. Intravenous formulations of antibiotics may contain additives and preservatives harmful to the lungs or not having appropriate osmolality, pH and particle size, which can cause airway irritation, cough and bronchospasm [66]. During nebulization, antibiotic liquid aerosols are generated by mechanical mechanisms like soft mist inhaler, human powered nebulizer or electrical mechanisms such as vibrating mesh technology, jet nebulizer and ultrasonic wave nebulizer [67]. Recent advances in nebulizer design have been reviewed elsewhere [68]. Conventional jet nebulizers generally have low drug delivery efficiencies and compared to the other types of nebulizers, noisy working and heavy weight are the biggest drawbacks of the jet nebulizer [69]. These issues have been improved by vibrating mesh nebulizers to produce aerosols with greater concentration of droplets and to reduce their administration time. As a consequence, minimal residual volume is exhibited, which in turn yields lower antibiotic waste, rapid output and enhanced drug delivery efficiencies [70-73]. Pulmonary drug delivery by nebulization can also be optimized by digital software regulation and performance feedback systems [74]. Table II enlists some antibiotics designed by nebulization.

3.2. Dry Powder Inhalation

Regarding the possible dosage forms for the pulmonary delivery of antibiotics, one can use a wide variety of formulations, such as dry powder inhalation (DPI). DPI formulations have been used for patient treatment for more than 60 years, but during this period the fundamental formation of DPIs has not changed significantly [75, 76]. DPIs have

Table II *Nebulized antibiotics and their clinical impact*

Therapeutic agent	Method	Characteristic excipient	Clinical/Biopharmaceutical impact
Ciprofloxacin	Liposome formulation by membrane extrusion method	Hydrogenated soy phosphatidylcholine/ cholesterol	Enhanced drug encapsulation/size/ stable release [75].
Ciprofloxacin	Liposome formulation by membrane extrusion	Polysorbate 20/hydrogenated soy phosphatidylcholine/cholesterol	Enhanced release rate [76].
Ciprofloxacin	Liposome formulation by thin film method	(DOTAP)*/ (DOPE)** (PC)***/ cholesterol	Decreased MICs [77].
Colistin	Dry film method	Dioleoyl-phosphatidylcholine	Enhanced release rate [78].

*1,2-Dioleoyloxy-3-trimethylammonium-propane **1,2-dioleoyl-sn-glycero-3-phosphoethanolamine *** phosphatidylcholine

become the first choice of inhaled formulations in European countries. DPIs are formulations containing micronized drug particles with an aerodynamic particle size of less than 5 μm [77]. For adequate deposition to reach the central and alveolar parts of the lungs, the optimal size of particles should be in the region of 1-5 μm. The most important approach in designing DPIs is that the time required for delivering each dose is short and even less than one-third of the time is needed for delivering the same dose for nebulization. This fact is expected to improve patients' adherence [78, 79]. DPIs of antibiotics are more stable, offer ease of administration and have less risk of microbial contamination than parallel liquid formulations [80-82]. DPIs have conventional application as a formulation of micronized drug in a carrier-based system [83]. Because the small particles (1-5μm) tend to stick with each other due to high surface free energy, carriers such as lactose, mannitol and trehalose are used for preventing the agglomeration of particles. These excipients reduce the surface energy, overcome cohesive forces and adhesive forces, and limit the flowability of API particles [83]. That is why the carrier-based system is being explored for surface modification and active targeting. By an appropriate use of the polymer or lipid carrier, the pulmonary drug delivery approach can result in interesting outcomes.

Lactose is the most typical and frequently used carrier in DPIs but because of clinical issues, lactose cannot be used for drug delivery to diabetic patients and people with lactose intolerance [84-87]. Mannitol, a hexahydric alcohol, has been frequently used as a carrier for aerosol drug delivery [17]. Mannitol is less hygroscopic than lactose and gives a suitable sweet aftertaste, which is a benefit over lactose and enhances the compliance of patients [88]. A therapeutic DPI aerosol for the treatment of CF and chronic bronchitis (BronchitolTM), approved by the FDA and the EMA, contains mannitol as a carrier system [89]. A DPI formulation for the inhalation of ciprofloxacin hydrochloride was prepared with different percentages of mannitol as a combination formulation. Mannitol improved mucous clearance in the respiratory tract while concurrently treating local chronic infection, chronic obstructive pulmonary disease and cystic fibrosis [90]. Trehalose dihydrate is a disaccharide non-reducing sugar and can be used as another carrier. A DPI of trehalose microparticles with low water content was successfully produced by the spray-drying technique [91]. Although trehalose leads to autophagy and can be used for the treatment of Huntington's disease, Parkinson's disease or tauopathies, it does not exhibit any benefit for the treatment of infections [92]. Moreover, DPIs of antibiotics usually have large therapeutic doses (e.g. between 10 mg and 100 mg of antibiotics), thus the carrier causes difficulty in the application of the DPI due to the increased powder volume and the scaled-down use of antibiotics via pulmonary dosage forms [93]. For about the last two decades, there has been significant research on the design of carrier-free systems for DPIs [83]. Applying a carrier-free system makes the delivery of a high dose of antibiotics to the lungs possible by limiting the amount of excipient [94]. Carrier-free formulations can be handled by coating particles with lipids, amino acids and polymers by the mechanofusion dry coating process [95,–98]. The drug deposition of DPI in the lungs is essentially controlled by its aerodynamic behavior. Currently, the aerodynamic properties of DPIs are being improved by changing formulation strategy and particle engineering [99]. These strategies are discussed in detail in Section 3.3.

3.3. Preparation methods for DPIs

3.3.1. Milling (top down)

Milling involves the breakdown of coarse large particles into fine particles by the use of mechanical force. Wet milling and dry milling are the common methods used in the production of pharmaceutical products. As the name indicates, wet milling involves the breakdown of large particles while they remain suspended in liquid medium. Dry milling may be sub-branched into various other forms of milling that do not require moisture content during the breakdown. Wet milling is often used for drugs which have a high residual moisture content [100]. The pharmaceutical industry also uses jet milling, also referred to as fluid energy milling, for most of the pharmaceutical dosage form designs. Jet-milled powders are highly cohesive because of the high surface energies of the particles. This problem can be resolved by adding excipients and carriers. However, this approach seems unfavourable for high-dose antibiotics. The great advantage of this method is that it does not require separation [101-103]. The process of milling can lead to a decrease in the particle size and moderately reduced crystallinity because of the production of amorphous form [104]. So particle engineering is a very important key factor for the production of carrier-free (or with minimum carrier) inhalable powders of antibiotics with good aerosolization behavior [105, 106]. Overall, the process of milling improves drug dissolution and solubility profiles.

3.3.2. Solvent evaporation method (bottom up)

The other process used commonly is the solvent evaporation method. It includes spray-drying, freeze-drying, spray freeze-drying, and supercritical fluid followed by rapid expansion. Spray-drying is a single-step particle formation process and is an appropriate way for particle engineering under a controlled manner for scale-up in industry. It is used for the production of dry powder from a solution, suspension and emulsion by rapid drying in the presence of a hot gas [107]. Amorphous and crystalline materials may be yielded by spray-drying depending on feedstock. This method gives better control over the particle size and shape, yielding powders with a narrow particle distribution and low particle surface energy. Furthermore, it creates possibility for the addition of excipients to promote the dispersibility of the powder, to enhance the stability of the formulation, to improve cellular uptake and to complete a formulation with modified drug release. Carrier-free DPI formulations of ciprofloxacin nanoplex were developed by spray-drying and spray freeze-drying methods. D-Mannitol and L-leucine were used as drying adjuvant and aerosol dispersion enhancer, respectively. Another example is the manufacturing of inhaled tobramycin (TOBI® podhaler®, Novartis) [108-110]. PulmoSphere of tobramycin can also be prepared by treating an emulsion under high-pressure homogenisation followed by spray-drying.

Different excipients have different effects on the mass, particle size, particle morphology and aerodynamic behavior of microparticles [111]. A mannitol–leucine combination resulted in better aerosolization behavior of the therapeutic agent, but mannitol exhibited some degree of recrystallization. A trehalose–leucine combination shows good potential to be used as excipient for the pulmonary delivery of potent antibiotics [109]. Although spray-drying is a conventional method to produce DPIs, the exposure of heat-sensitive antibiotics, e.g. penicillin, to the high temperature of the spray dryer (>100℃) is not appropriate. The nano spray dryer provides very adequate results for the formulation of heat-sensitive materials in submicron particles, with high yields (70% to 90%) related to the conventional spray-drying method [112]. Freeze-drying works by freezing the therapeutic agent and then decreasing the pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase [113]. Freeze-drying has been considered as a good technique to enhance the long-term stability of the microparticles and nanoparticles of antibiotics [114].

The worldwide rise in mortality rates because of antibiotic resistance turned out to be the toughest challenge to modern medicine and therapeutic agents [115]. Monotherapy with a single antibiotic may lead to the development of antibiotic resistance due to newly discovered pathogens, which cause resistance to a broad spectrum of antibiotics [116]. Hence, combination therapies, containing different types of antibiotics, have been introduced to inhibit the development of drug resistance [117]. Antibiotic combinations should be according to the synergistic effect of antibiotics and should avoid interaction [118]. Cospray-drying is the method which can assist in such combination therapy to achieve the desired effect. The cospray-dried combination of ciprofloxacin and doxycy-

Table III *Differences between conventional and QBD approaches*

Characteristic	Conventional	QbD
Pharmaceutical development	Univariate experiments	Multivariate experiments
Manufacturing process	Fixed	Flexible
Process control and control strategy	Slow and by initial intermediate and end product testing	Actual time, risk-based controls shifted upstream
Product designation	It is based on batch data	based on desired product achievement (safety and efficacy)

cline hydrochloride (1:1) is suitable for inhalation and highly effective against *Staphylococcus aureus*, *P. aeruginosa* and *Streptococcus pyogenes* [119]. A formulation consisting of highly porous nanoparticles loaded with tobramycin surrounded by a matrix composed of amorphous clarithromycin, with a median particle size of about 400 nm, was synthesized by high-pressure homogenisation. Interestingly, the results showed that the formulation of the combination of two antibiotics enhanced powder dispersion during inhalation. Local drug deposition profiles were almost similar for the antibiotics and reached the target site concurrently. The dissolution rate revealed that tobramycin and clarithromycin dissolve with ease in the lungs [93]. A formulation comprising ciprofloxacin hydrochloride and gatifloxacin (fourth generation of fluoroquinolone), prepared by the spray-drying method, showed a synergistic antimicrobial effect in the lungs [120].

4. Novel DPI formulation strategies and carriers for inhaled antibiotics

4.1. Preformulation and Quality by Design approach

The majority of research and innovation for pulmonary dosage forms of antibiotics does not achieve scale-up and marketing. The main reasons are the lack of feasible process, the inappropriate way for the efficient and effective control of changes, the inability to achieve reasonable product quality, the high cost with a low yield, the inability to predict effects of scale-up on the final product, the inability to analyze or understand reasons for manufacturing failures, and the large number of batch failure. Hence, Quality by Design (QbD) is necessary before every laboratory research, new formulation, particle engineering and powder formulation [121-124].

The pharmaceutical QbD is a systematic pathway for the development of a new formulation, which begins with a predefined formulation and indicates product and process understanding and process control, based on quality risk management [125]. QbD appears to enhance the assurance of safe and effective drug supply to the patients, and also attempts to significantly improve manufacturing quality administration. QbD principles have been used to regulate product and process quality in industry and have been approved by the FDA for the discovery, formulation and development of drugs [126]. Table III identifies some of the characteristic differences between conventional and experimental design QBD approaches.

So QbD ensures better design of products with fewer problems in manufacturing and allows for the better understanding of how APIs and excipients affect manufacturing. It also leads to a reduction in the overall costs of manufacturing, thus speeding up the process of approvals and accelerating scale-up production [127]. The specific design of the inhaler is very critical in achieving acceptable airflow to deposit the drug into the therapeutically effective region of the lungs [128]. DPI dosage form properties can be controlled by adjusting the particle size, size distribution, particle density, particle morphology and shape [129-131]. The Ishikawa diagram in Figure 3 illustrates the parameters influencing the quality of DPI products in general, assembling all the influencing parameters of the aimed DPI product [97].

An amikacin product for inhalation in CF patients was manufactured by spray-drying the pure drug, and the formulation exhibited great respirability and flowability. An experimental design was applied on the process in relation to six Critical Quality Attributes (CQAs) of the finished product and five Critical Process Parameters (CPPs). The application of the experimental design was set up to achieve amikacin powders with both emitted dose (ED) and fine particle dose (FPD), completely with high regulatory and scientific references [132].

The dry powder formulations of ciprofloxacin hydrochloride were prepared by the spray-drying method following the QbD approach. An advanced quality management method was used to predict the final quality of the product in relation to the QbD-based theoretical preparatory parameters. Dry powder inhalation formulation tests

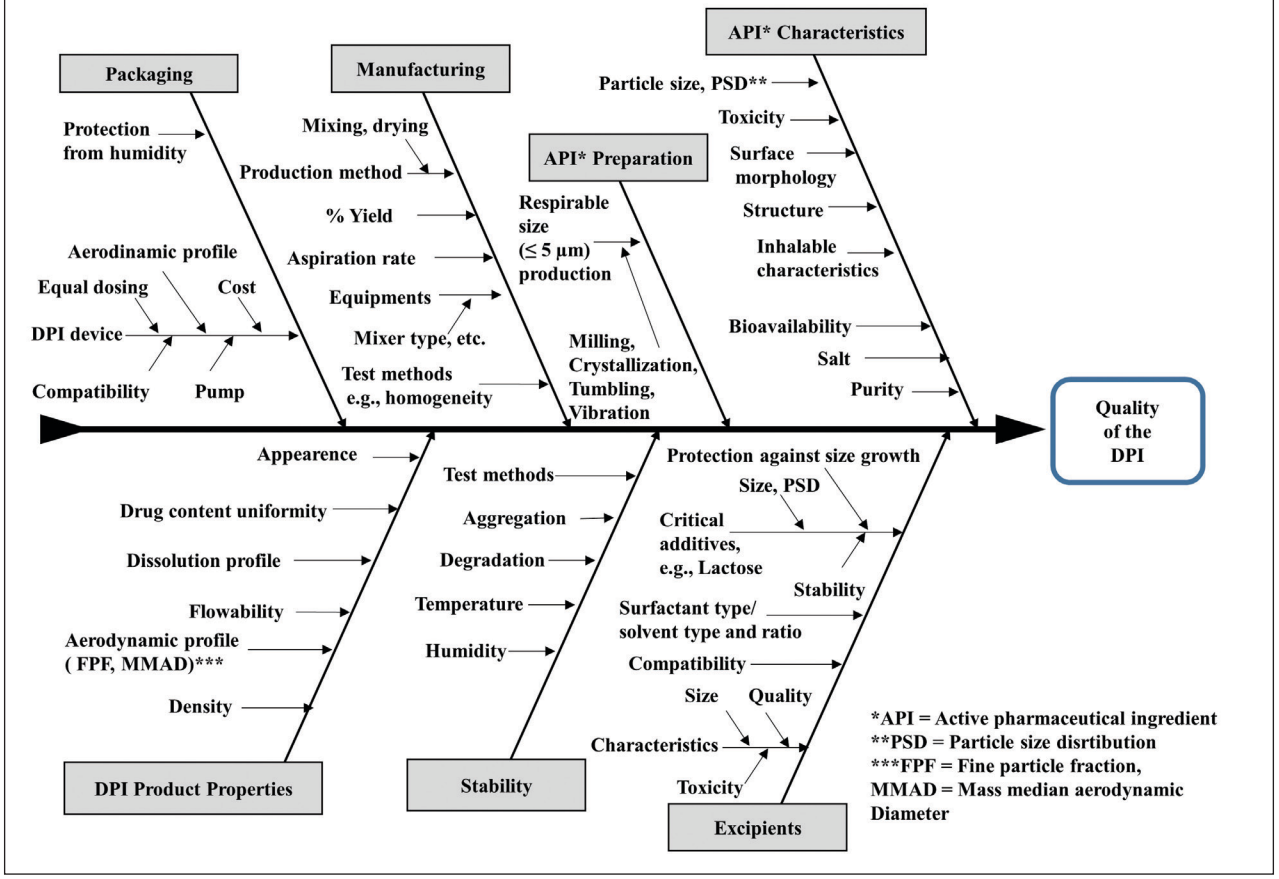

Figure 3 *Ishikawa diagram for DPI formulation in general*

were then successfully performed in practice [133].

4.2. Novel formulations and carriers for antibiotics

4.2.1. Microparticles

DPI formulations are usually comprised of micronized drug powder. One of the highest significant upgradations, in powder technologies from the micronization of large drug crystals into a respirable range for use in DPIs, is enhancing their dispersibility by the reduction of interior adhesive forces in the crystals [105]. Pharmaceutical industries have high demand for crystalline pharmacons. Most products for pulmonary dosage forms in the market are being manufactured in the crystalline state.

Crystalline drugs exhibit more stability; and for formulation development, thermodynamically stable polymorphs are selected. Salts are selected for their better solubility, purity and crystallinity relative to the neutral form [134]. For example, a DPI formulation was prepared with the sonicated solution of ciprofloxacin in acetone because of the very low solubility of neutral ciprofloxacin. In this formulation, L-leucine was used as a characteristic excipient [135]. In another DPI formulation, ciprofloxacin hydrochloride was used and the formulation preparation did not require a toxic organic solvent and a complicated method due to the high solubility of the salt form in water. A great advantage of the second formulation is that L-leucine can dissolve in water easily, too. In both cases the DPI showed excellent aerodynamic behavior with a fine particle fraction (FPF) value of more than 80% [133].

The conventional method of drug powder formulation in the microsized range involves crystallization followed by milling to reduce the particle size and to attain the suitable size. This method is not an appropriate method as it implies incomplete control over the particle size, size distribution, particle morphology and crystallinity. Mucoadhesive microparticles are able to swell and hydrate after deposition in the lung epithelial cells [136, 137]. The encapsulation of ciprofloxacin in chitosan is one such example, as the polymer has

swelling properties along with biodegradability and biocompatibility characteristics, and antibacterial and anti-inflammatory properties. Additionally, these swelling microparticles possess bioadhesive properties, promoting adhesion to the pulmonary system and enhancing antibacterial effect [136, 138].

For the microparticles to maintain sustained local antibacterial effect, they should avoid phagocytosis by alveolar macrophages. The particle size range that is optimal for pulmonary inhalation (1–5 μm) is also optimal for phagocytosis [139, 140]. Large porous microparticles, with low density but large geometric diameters, display ideal lung deposition profiles and can overcome phagocytosis challenges [141-143]. Spray-drying is generally used with different excipients like dipalmitoyl-phosphatidylcholine (DPPC) and albumin to produce large porous microparticles [144, 145]. Also, large porous microparticles can be produced by treating solid microparticles with supercritical CO_2 [146, 147]. Another interesting method for the production of large porous microparticles is the application of ammonium bicarbonate as an effervescent porogen, which decomposes into ammonia and carbon dioxide in an acidic aqueous solution or at high temperature [148].

Porous particles of tobramycin and ciprofloxacin produced by the emulsion method followed by spray-drying exhibited enhanced and satisfying flowability and aerosolization performance [149, 150]. A simple double-emulsion method using poly(DL-lactide-co-glycolide) polymer resulted in large porous biodegradable microspheres of capreomycin for pulmonary drug delivery. The morphology of particles displayed a highly porous interior and an outer rough surface [151].

4.2.2 Nanoparticles

Nowadays, nanoparticles are being widely investigated for antibiotic inhalation therapy [152], however, the formulation of nanoparticles for drug delivery application came to the fore in the 1960s [153]. The considerable advantage of nanoparticle formulations is that they improve the solubility and dissolution rate of water-insoluble antibiotics [154]. As an example, the nanoparticles of ciprofloxacin exhibited a speedy dissolution profile compared to the supplied ciprofloxacin powder. Besides, this formulation of nanoparticles of antibiotics enhanced the Minimum Inhibitory Concentration (MIC) and antibacterial activity. It was also observed that amikacin nanoparticles exhibit MIC and a bacteriostatic effect against *P. aeruginosa* compared to less than half of the values for free amikacin [155, 156]. Due to their small size and large surface area, the nanoparticles of the antibiotic showed significant and enhanced aerodynamic behaviour. An example of tobramycin nanoparticles can be noted where the formulation exhibited an FPF of 61% compared to the microparticles of tobramycin with an FPF of 36% [157].

On the other hand, nanoparticles act as foreign materials, with special physiochemical properties, in human bodies and are recorded to have severe adverse effects on the lungs, like inflammation, fibrosis and mutations along with oxidative stress. Further, these damages could cause pulmonary diseases and diseases in the other parts of body [158]. Inhaled nanoparticles can be exhaled because of their extremely low mass. These problems have been rectified by formulating nanoparticles into inhalable microparticles into a matrix or carrier system. These matrices can be synthetic polymers such as PVA, PVP and PLGA; amino acids like L-leucine; or polysaccharides such as chitosan and sodium hyaluronate [159-161].

4.2.3. Solid lipid microparticles and solid lipid nanoparticles

The incorporation of lipid into formulations brought about the development of porous particles with low density [162, 163]. There are various methods which have been reported for the synthesis of solid lipid microparticles (SLM) and solid lipid nanoparticles (SLN) [164]. Some of these methods are double emulsion solvent evaporation with freeze-drying [165], high pressure homogenization followed by spray-drying [166-168], melt emulsification followed by spray-drying [169], melt emulsification followed by freeze-drying [170, 171] and simple spray-drying [144]. SLNs usually have a spherical shape consisting of a solid lipid bulk stabilized by a surfactant. Biological membrane lipids such as phospholipids, and sterols (cholesterol) can be applied as stabilizers [172]. The most important advantages of SLNs from the pulmonary perspective include the possibility of large-scale production and ability of the incorporation of lipophilic and hydrophilic drugs, lack of biotoxicity of the carrier, high loading capacity, drug target delivery and controlling drug release [173].

Table IV *Different DPI formulations with their therapeutic outcome*

Therapeutic agent	Method	Characteristic Excipient	Particle size	Resulting therapeutic outcome
Ciprofloxacin and doxycycline	Spray-drying method	PVA*	Microparticle	Controlled release antibiotics [94].
Levofloxacin	Nanoprecipitation/emulsification–solvent evaporation methods	PLGA**/PCL***	Nanoparticle	Improved antibacterial efficacy [188].
Levofloxacin	Emulsification–solvent evaporation method	PLGA**/phosphatidylcholine	Nanoparticle	Improved antibacterial efficacy [189].
Tobramycin	Emulsion/solvent diffusion method	PLGA**/PVA*/ chitosan/alginate/ lactose	Nanoparticle	Increased encapsulation efficiency/release rate/lung deposition pattern [190].
Amikacin	Solid-lipid coated by solvent diffusion method/ freeze-drying	Sucrose/ Dextrose/Mannitol	Nanoparticle	Long-release term/antibacterial efficacy [159] [160].
Ciprofloxacin	Sonicating/freeze-drying	L-Leucine	Nanoparticle	Increased the dissolution rate/ improved aerodynamic properties [139].
Ciprofloxacin hydrochloride	Spray-drying	L-Leucine/PVA*/ Cyclodextrin	Microparticle	Enhanced the aerodynamic behaviour [137].
Tobramycin	High-pressure homogenisation/spray-drying	Sodium glycocholate	Mixture of micro- and nanoparticles	Enhanced lung deposition [161].
Ciprofloxacin	Self-assembly method	Chitosan/PEG	Loaded nanoparticle in micro hydrogel particles	Suitable aerodynamic characteristics/sustains drug release [140].
Ciprofloxacin	Emulsion/spray-drying	_____	Microparticle	Enhanced tolerability assessments [153].
Ciprofloxacin	Anti-solvent precipitation method/spray-drying	_____	Microparticle	Enhanced aerosol performance [191].

*Poly-vinyl alcohol **poly(lactic-co-glycolic acid) ***Polycaprolactone

4.2.4. Liposomes

Discovered by Dr. Alec Bangham in 1961 [174], liposomes seem to be a relevant and useful choice for pulmonary drug delivery considering their preparation from components compatible with the lungs, with a good safety profile. Arikayce is the first liposomal preparation clinically approved for pulmonary administration. No marketed inhaled liposomal antibiotic preparation was available previously [175]. Liposomal formulations of inhaled antibiotics are considered to be sustained drug delivery systems due to their low and slow solubility. These formulations prolong the action of drug in the infectious part and increase the antibacterial effect. On the other hand, the sustained release of antibiotics minimizes dosing frequency and thereby enhances patient compliance. Liposomal antibiotics can also act as targeted drug delivery systems [176]. The encapsulation of drugs in liposomes also reduces the occurrence of local irritation as that caused by traditional pulmonary dosage forms. Overall, these benefits of liposomal formulations make them an appropriate drug delivery system for antibiotics. The surface-mannose modification of liposomes with mannose promotes the active targeting of macrophages with mannose receptors and provides efficient aerosolized liposomal delivery [177]. Liposome formulations can be administered in a liquid dosage form, e.g. nebulizer. However, some solid preparations prepared by spray-drying or spray freeze-drying can be designed as DPIs [178].

Liposomes are formed immediately when lipids are hydrated in contact with water and then dried afterwards to form spheres. Generally, in a large scale-up process, lipids are first dissolved in an appropriate solvent (mixture of water, ethanol and the other organic solvent in a different ratio) and then rotatory evaporation removes the solvent. A thin layer of lipid film is formed on the wall usually in multilamellar vehicles (MLVs) [179-181]. Another method is the ethanol injection method, in which liposomes are formed after the

injection of the organic phase into the aqueous phase and then by applying diafiltration or ultrafiltration to remove the excess solvent [182, 183]. The possibility of encapsulating hydrophilic and lipophilic drugs and easy scale-up are major merits of this method.

A few DPI formulations with their therapeutic outcomes are mentioned in Table IV.

5. Patient History

The efficacy and tolerability of nebulized antibiotics in trials remain low and only involve a single center or are confounded by inadequate patient enrollment, poor methodology, failures in standardizing or reporting delivery methods, and particle sizes. Different studies have used different doses or formulations as well as differing patient cohorts. Hence, there is no standardized technique for the administration of a given aerosolized drug. These factors make the comparison of efficiency and tolerability difficult and pose challenges when trying to standardize this method of treatment and in establishing best practice [184]. Studies of inhaled antibiotics targeting non-CF pathogens for suppression, eradication or prophylaxis are scarce [75], and no inhaled antibiotics are approved for lungs in non-CF infections, including COPD, melioidosis, pneumonic plague, anthrax, Q fever, tularemia, and for patients with other infections, including non-tuberculous mycobacteria. Despite the need, limited ongoing studies are observed for the dry powder form of vancomycin assessing the efficacy and safety of suppressive therapy for methicillin-resistant Staphylococcus aureus (MRSA) infection. The only recommended prophylactic strategy available is the chronic prophylaxis to prevent the acquisition of S. aureus, which is used primarily in the UK [185].

6. Future perspective and conclusion

Inhalable powders in the form of nanoparticles have potential as a treatment option of respiratory tract infections. Targeted delivery is possible by the optimization of formulation parameters, and new nanoparticle formulation strategies that may enhance safety, stability, dispersion and deposition.

Liposomal formulations of inhaled antibiotics are advanced drug delivery systems designed for sustained drug release and targeted drug delivery to the lungs; nevertheless, low stability and difficulty in liposomal DPI production are notable issues. In this field new techniques and strategies are necessarily required to overcome the challenge of instability of most liposome formulations.

In the development of combination therapy, it is possible to create novel technological methods to design a combination of antibiotics in which each particle can have several layers made of different antibiotics with different bactericidal activities. In this way, resistance by bacteria can be reduced, and therefore a new dimension to antibiotic treatment can be explored.

Inhaled antibiotics for the treatment of respiratory tract infections have a great and long history; however, these therapies focus on CF patients. Right now, there is no academic indication for using inhaled antibiotics for the treatment of non-CF patients. Hence, prescriptions for patients with non-CF respiratory infection will continue to be based on oral or parenteral dosage forms until scientific evidence from progressing clinical trials become available. Literature data concerning non-cystic fibrosis patients in the next years will explain much. Pulmonary dosage forms of antibiotics show interesting results due to high drug concentrations in the respiratory tract with minimum systemic drug exposure. However, the formulation of antibiotics for pulmonary dosage forms is relatively complicated. Antibiotics are administered in higher doses than the other therapeutic agents for asthma or other inflammatory diseases. DPIs have also been favored in recent years for the delivery of inhaled antibiotics. The particle engineering technique is a key factor to improve inhalable formulations that are able to deliver the drug with advanced therapeutic effect. Advanced particle engineering techniques are also being employed to revise the manufacturing of DPI formulation for delivering antibiotics. Pulmonary delivery systems for the treatment of viral lung infections are completely absent, and this area should be explored to develop potent antiviral therapies.

Disclosure

The authors report no conflict of interests in this work.

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Formulation and characterization of pulmonary drug delivery systems

SABRINA MAGRAMANE, ZSÓFIA PÁPAY, BÉLA TURBUCZ, ISTVÁN ANTAL*

Department of Pharmaceutics, Semmelweis University, Hőgyes Str. 7., Budapest, H-1092

**Corresponding author: István Antal*
E-mail: antal.istvan@pharma.semmelweis-univ.hu

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Abstract
The inhalation therapy is one of the oldest drug delivery methods known. The significance of inhalation can be understood notably through its remarkable history. The goals of this review are to explore the pulmonary drug delivery, its significant relevance and various advantageous properties, particularly due to the physiology of the lungs. The drug delivery into the lungs can be provided by several inhalation instruments presently accessible on the market such as nebulizers, MDIs, and DPIs. Supplementary devices suchlike spacers for instance are also available in order to optimize the therapy results. The efficiency of these devices depends on several parameters of the formulation used, as well as its deposition in the lungs. Therefore, this review focuses on the meticulous testing performed on both the formulation and the device carrying it in the interest of insuring safety, quality, and efficacy of the final product. Ultimately, the pulmonary drug delivery represents a substantially advantageous alternative route of administration to obtain a systemic effect as well. This review aims to the better understanding of the development of pulmonary dosage forms and its complex process which requires extensive considerations and thorough optimization.

Keywords: *pulmonary drug delivery, inhalation, lung deposition, particle size, inhaled formulations.*

1. Introduction – Historical review of inhalation for drug delivery

The substantial progression witnessed in the development of inhalation devices may indicate that pulmonary drug delivery is a novel route of administration. However, the use of treatments through inhalation for therapeutic intents has existed for thousands of years and has been practised in numerous civilizations (Figure 1). Four thousand years ago, in India, pulverized jimsonweed (*Datura stramonium*) and angel's trumpet (*Datura ferox*) were mixed with natural ingredients such as pepper or ginger and smoked for their bronchodilating therapeutic properties, due to their alkaloids content [1, 2]. Apart from that, the oldest known mention of the use of inhalation for therapeutic purposes dated back to 1554 BC and was found in the *Ebers papyrus*. In this Egyptian papyrus scroll, a depiction of patients who seem to have trouble breathing can be found. Black henbane (*Hyoscyamus niger*) is put on hot bricks, and the vapour generated is respired by the patients through a stem of reed attached to a pot placed

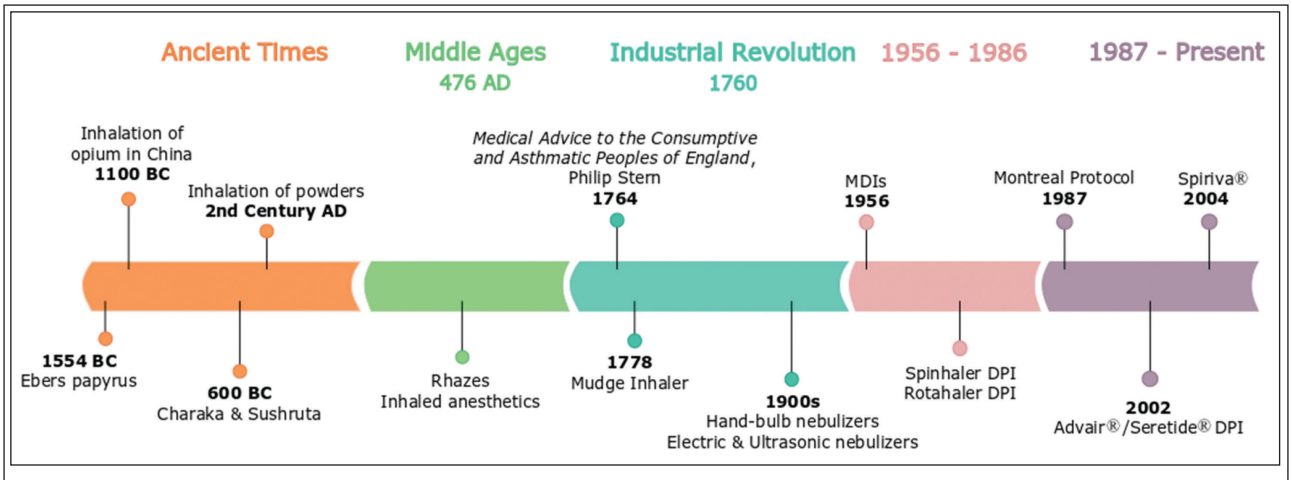

Figure 1 *Timeline of the History of inhalation therapy*

over the burning plant. *Hyoscyamus niger*'s remedial properties come from its tropane alkaloids contents, particularly atropine and its anticholinergic properties [3].

In 1100 BC, opium was smoked using pipes and incense burners for both therapeutic and recreational purposes in China. This marks one of the most notable uses of the pulmonary route of administration, although Avicenna thoroughly detailed opioids' toxicity and dissuaded their usage [3].

In 600 BC, a description of the treatment of asthma by therapeutic inhalation was detailed in the scriptures of Charaka and Sushruta (notorious Indian physicians). *Datura stramonium* (which contains atropine) was used against asthma symptoms, in the form of a cigarette or a pipe. In his writings, Charaka also describes steam inhalation and cigars (made of several natural ingredients such as the paste of turmeric) as asthma therapies [3].

In the second century AD, Galen suggested the use of powders of myrrh and nutgall through inhalation to reduce nasal and chest symptoms [3].

From the Middle Ages most therapies were based on the methods developed during the Ancient Times such as the smoking of opium or *Datura stramonium*. One of the most groundbreaking approaches of the Middle Ages was made by the Arab physician Rhazes. He developed a liquid mixture of narcotic plants: opium, mandrake, and henbane. A sponge was then soaked in the solution and left to dry. Before surgery, the sponge was humidified and placed on the patient's mouth and nose. The patient would then inhale the fumes generated by the sponge. These vapours produce an anaesthetic effect [3].

With the start of the Industrial Revolution (1760-) came significant innovations, notably in manufacturing procedures. The respiratory drug delivery acquired a particular attention when the English physician Philip Stern declared that "the only possible way of applying medicines directly to the lung is through the windpipe". The inhalers created by Stern and English physician John Mudge amplified the popularity of the treatment of asthma through the inhalation of pharmaceutical drugs [1, 4, 5]. As a consequence, various ceramic inhalers were developed.

When it comes to drug inhalation, the therapeutic aerosol had to be prepared by either the physician or the patient himself. However, during the Industrial Revolution, the discoveries and advances made allowed for the aerosol to be made by a third party and even to be produced on a large scale. Furthermore, novel entities and techniques were designed, allowing for the active ingredient to be isolated, and its safety and potency to be improved. The Industrial Revolution era also marks the launch of nebulizers and early versions of dry powder inhalers (DPIs) [3].

The early 1900s were marked by the popularity of hand-bulb nebulizers delivering adrenaline chloride for its bronchodilating properties. In the 20th century, new types of nebulizers were developed: the electric and the ultrasonic nebulizers [1].

The period between 1956 and 1986 brought the advances of pressurized metered-dose inhalers (MDIs) and later the dry powder inhalers which are still broadly used presently. The introduction of metered-dose inhalers (MDIs) started in 1956 [1, 2], among the first approved MDIs was the Medihaler-Ergotamine, a device delivering ergotamine tartrate for migraine therapy, which illustrates the early interest for the therapy of systemic conditions through the pulmonary route of administration [6]. Supplementary devices were also developed to ameliorate the efficacy of the inhalers and to facilitate patient coordination. Two DPIs, in particular, marked this period: the Spinhaler® and the Rotahaler®. The Spinhaler® delivered 20 mg of sodium cromoglycate [1] from a gelatin capsule which is later on pierced by the device. The formulation aspects of this inhaler shaped the present formulation development of DPIs. The Rotahaler® delivered albuterol from a capsule which is later halved by the device [3].

The latest period since 1987 (marked by the signature of the Montreal Protocol eliminating CFC propellants) is characterized by a recordbreaking advancement in the pulmonary drug delivery with the rise of yearly sales from $7 billion in 1987 to $36 billion in 2014 [3]. In 2002, the introduction of Advair®/Seretide® delivering salmeterol xinafoate/fluticasone propionate for the treatment of asthma resulted in the first multibillion- dollar DPI. Another example is the Spiriva® inhaler launched in 2004, delivering tiotropium. It was the first inhalation drug intended for the treatment of COPD (Chronic Obstructive Pulmonary Disease), which at the time was an inefficiently treated disease [6].

2. Advantages of pulmonary drug delivery

The pulmonary delivery is an interesting drug delivery route for locally acting treatments of lung diseases such as asthma. However, owing to the scientific development of pharmaceutical formula-

tions and inhalation devices, pulmonary drug delivery plays an important role in delivering drugs systemically and treating systemic conditions such as diabetes, certain autoimmune diseases and some types of cancer [7]. Pulmonary delivery can be obtained via oral or nasal routes. However, higher drug deposition can be achieved with the former way. This can be attributed to the physiological structure of the human lungs. The respiratory tract has two distinct parts by function: the respiratory zone and the conducting airways [8]. According to Weibel's lung model – which is the simplest and widely used model – there are 23 so-called generations (G) and the trachea (G0) (Figure 2) [9]. The evolution starting from the trachea to the alveolar ducts can be described by decreased tube length and cross-sectional tube area, yet an increased number of tubes. In the lumen, the trachea has a width of approximately 2.5 cm while the alveolar ducts possess a diameter of only 0.2–0.5 mm. Thereby the airways have a surface area of about 2.5 m^2, and that of the alveoli is about 100 m^2. The mouth and nasal cavities, pharynx and larynx, also belong to the conducting zone and are responsible for carrying the gas to the site of the gas exchange, as well as filter, warm up and humidify the inspired air. The gas exchange occurs from the respiratory bronchioles (G17) to the alveolar sacs (G23) [8, 10].

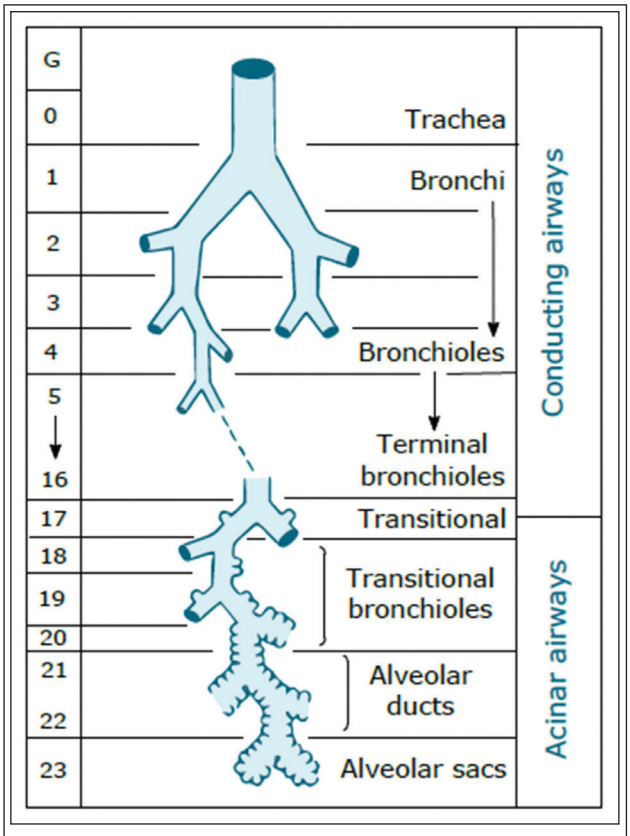

Figure 2 *Weibel's lung model (based on [8])*

Due to their physiological properties, namely a large surface area beneficial for absorption (100 m^2), the lungs possess a unique platform for drug delivery, whether locally or systematically. This also allows a rapid onset of action of drugs. There are no food effect or pH problems, and the first-pass metabolism can be avoided. Consequently, a decreased effective dose can be used, and fewer side effects could occur.

3. Inhalation devices

The painless drug delivery - compared to injections - could increase the patient's compliance. However, it is necessary to inform them about the proper manipulation of the inhalation devices. Up to 50% of the patients misuse the inhalers due to the incoordination of the inhalation with the actuating element. Three main types of devices are available on the market: nebulizers, pressurized metered-dose inhalers (pMDIs) and dry powder inhalers (DPIs) (Figures 3-5) [8].

Aerosols are solutions or suspensions, where the active ingredient (in a liquid or solid- state) is suspended in a carrier gas, intending to deliver the drug to the alveoli. *Nebulizers* can transform the solution or suspension into small droplets based on different working mechanisms (jet, ultrasonic and mesh). They consist of a medication reservoir, a baffle compressor, a mouthpiece and a facemask. The general disadvantages of nebulizers are the high cost, difficulty in cleaning the device and the fact that drug wastage could occur. Moreover, electricity is necessary for the ultrasonic devices. On the other hand, they are easy to use, the contamination could be reduced, and they are more efficient when it comes to delivering active ingredients which cannot be delivered with the other devices.

Pressurized metered dose inhalers (pMDIs) are the most frequently prescribed devices for asthmatic or COPD patients. Single-dose and multi-dose devices are accessible on the market, consisting of drug concentrate, propellant, metering valve and actuator. The main benefit of pMDIs is that the aerosol is formed by atomizing the extremely volatile propellant which is used to deliver an exact dose within a short treatment time. They are small and portable with a dose counter; therefore the patient can easily follow the therapy.

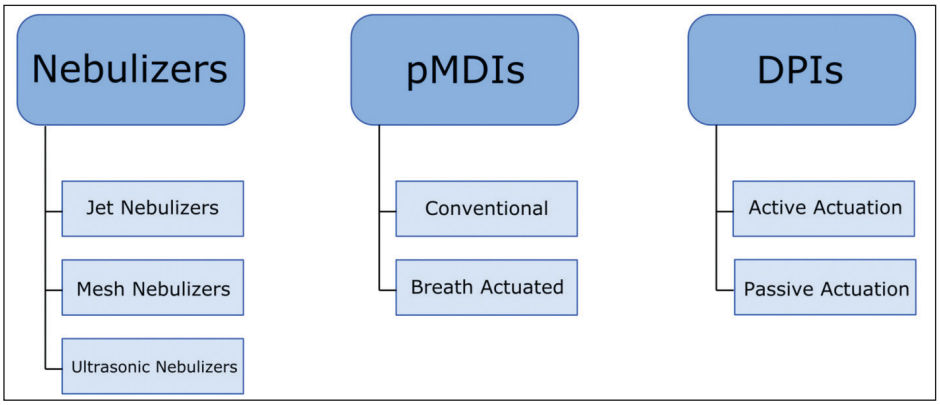

Figure 3 *Types of inhalation devices*

Figure 6 *Respimat® Soft mist inhaler*

Figure 4 *Examples of conventional pMDIs: Ventolin Evohaler, Serevent Evohaler, Foster, and Atimos*

Figure 5 *Examples of passive DPIs: Breezhaler, Symbicort Turbuhaler, Pulmicort Turbuhaler, Handihaler, Foster Nexthaler, Ellipta, and Seretide Diskus*

Soft Mist inhalers (SMIs) are MDIs, which are propellant-free. In order to produce an inspirable aerosol from a solution, the liquid dose needs to have a suitable droplet size. This can be achieved through two main methods: a first approach is the use of vibrations created by electrical energy (ultrasonic and piezoelectric devices). Another method would be pushing the solution through a nozzle using a mechanical force. An example of that is the Respimat® SMI (Figure 6), which functions by using the mechanical energy of a string compressed by the patient [11, 12].

Another example of SMIs is the AERx Essence® (Aradigm Corp.) which uses a breath-actuated piston system to push the solution through a nozzle arrangement, and a heating system to decrease the droplet size. A small screen incorporated to the device provides visual feedback to the patient. AERx Essence® is used for the pulmonary administration of insulin [12-14].

Dry powder inhalers (DPIs) are propellant-free devices, containing fractionated and a micronized powder formulation of drugs. When a patient uses their inhaler, their inspiratory airflow constitutes the principal driving force for delivering breathable particles in dry powder to the deep lung. DPI devices contain the powder formulation, a dose-measuring system and a mouthpiece. The main advantages are that they are propellant-free, portable and require a short treatment time. However, the main disadvantages could be the dependence on the inspira-

tory flow of the patient and the particle aggregation due to humidity [8, 10]. A study using an *in-vitro-in-silico* procedure was performed on several DPI formulations placed in a highly humid environment. This study examined how these DPI formulations could influence a treatment when placed in a high humidity environment, alongside patients' wrong storage of the inhalers. The DPIs used were Easyhaler® and Novolizer®, both containing budesonide and lactose. It concluded that formulations with smaller particle size and a higher quantity of excipient fines are more likely to be sensitive to humidity, which makes their efficiency inconstant after their storage [15]. This is particularly pertinent and problematic, considering that a significant amount of patients store their inhalers in unsuitable and highly humid conditions [16]:

- 42% of patients store their inhaler in a bathroom
- 21% in their pocket or a handbag

Concerning patients' errors, there are current trends which aim to face this challenge, such as smart devices. These devices are developed using electronic monitoring systems that can connect to other devices or even to an internet network. The goal is to decrease the errors made by both the patients and the devices, as well as to enhance the patients' compliance with inhalation therapy. These devices possess the ability to track the patients' adherence, as well as their use of the inhaler. The set goals are reached through characteristics such as a reminder which prompts the patient to take the dose, or even by providing the patient with a guide displayed on the inhaler's screen explaining the use of the inhaler step by step [17]. There are two different types of smart inhalers: "add-on" devices and "originally integrated" devices. An example of the originally integrated devices is the 3M™ Intelligent Control Inhaler by 3M™ Drug Delivery Systems [18].

4. Appropriate inhaler use and supplementary devices

Managing respiratory conditions such as asthma and COPD depends significantly on the proper use of the inhaler provided for the therapy. Incorrect use of the device or a faulty inhalation routine can reduce the drug delivery and therefore affect the disease control. A study conducted in France by Molimard et al. showed that out of over 3800 outpatients, 76% made a least one mistake while using a pMDI [19].

Even with the vast choice of devices available on the market and their level of precision, delivering an accurate dose can still be challenging sometimes. Moreover, when incorrectly using a pMDI, a poor drug deposition in the lungs occurs, resulting in a high oropharyngeal drug deposition [20-22]. Consequently, spacers (also referred to as holding chambers or extension devices) can be used. Spacers are supplementary devices which improve the performance of an inhaler. They work by keeping the medication in an enclosed space during the inhalation. The use of a spacer added to a satisfactory inhalation technique can improve the drug delivery by up to 5-10% [23]. A study organized in India showed that out of 300 patients, 247 (82.3%) made a mistake while using their inhaler. Out of these 247 patients, the highest number of errors was noted in patients using MDIs (94.3%). However, patients who used a spacer with their MDIs resulted in a smaller percentage (78%). Some of those errors were: "Inhaler not shaken" (40%), "Long delay before inhalation" (36%), and "Stopping inhalation as the device is fired" (32%) [24]. Thus, spacers not only reduce oropharyngeal deposition, but they also decrease the accuracy demand for the actuation and inhalation while using a pMDI on its own [25]. This is particularly advantageous in infants and children since they are unable to produce an accurate inspiratory manoeuvre or refuse to cooperate. It is also recommended to use facemasks, especially in infants [22, 23]. Moreover, the use of spacers and facemasks is also helpful and convenient for patients who require medical assistance, such as elderly patients with COPD and cognitive impairment [25, 26]. It has to be noted that spacers and facemasks are likely to be exposed to contamination by microorganisms. Since they are exposed and get in contact with mucous membranes, it is recommended they undergo cleaning, disinfection, rinsing, and air-drying after each use [27]. However, this cleaning procedure required after each use is not executed nor supported or endorsed by the manufacturers and the instructions leaflets they provide. Even so, it is advisable, at the very least, not to share spacers between patients [28].

In veterinary medicine, - proper use of spacers and facemasks can be observed to improve the therapy by optimizing the actuation. Since animal and human facial characteristics are not similar, a facemask constitutes an essential junction between the animal and inhalers such as nebulizers or

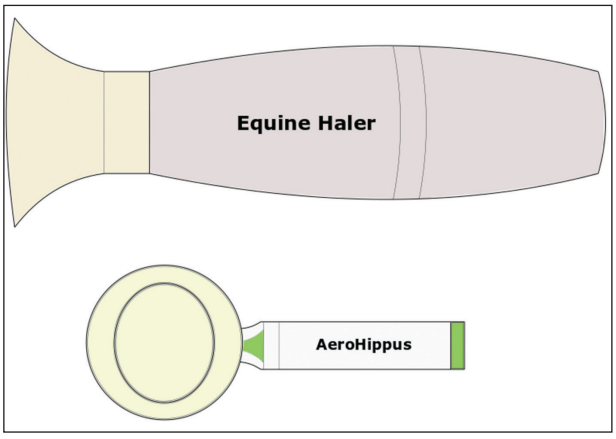

Figure 7 *Sketches of the veterinary Equine Haler and AeroHippus devices*

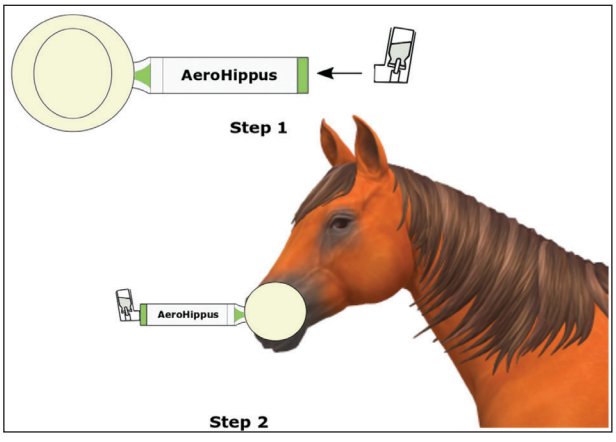

Figure 8 *Inhaling mask for horses*

pMDIs [29]. Moreover, contrary to humans, it is quite troublesome to control the breathing pattern of animals. In horses, for example, it is inconceivable to inquire a voluntary deep breath. Using an extension device such as spacers and facemasks allows the adaptation of commercially available human pMDIs to horses. Nebulizers could also be used, but unlike pMDIs with spacers, nebulizers permit the deposition of only small amounts of a drug in the equine lungs. In this regard, the utilization of pMDIs with a spacer in the veterinary practice is thought to be a great advance [30]. In case the horse reacts to the sound of the pMDI's actuation, there is a possibility for the inhaler to be actuated in the spacer, away from the horse's face. The chamber can then be placed on the horse's face, held up to the nostril, until the next inhalation. Examples of inhaling devices used for horses are Equine Haler and AeroHippus. (Figures 7 and 8) [30].

5. Inhalation as an alternative delivery route

5.1. Prospects and innovative developments

Inhalation as a delivery route finds its pertinence in the advantageous properties of the lungs (high permeability, large surface area). Moreover, an elevated local drug concentration can be obtained through pulmonary delivery, which is then rapidly absorbed through the alveoli, thus allowing a fast onset of action while at the same time still keeping systemic adverse effects to the minimum. The use of inhalation prevents specific gastrointestinal issues such as food effects, gut irritability, undesired metabolites, and poor solubility [31]. Furthermore, due to the absence of the first-pass metabolism, inhalation constitutes a great alternative to drug formulations, which cannot be delivered orally [32]. In addition, it also represents an excellent substitute for drug formulations, which are administered through an invasive method. Numerous macromolecules present in different stages of clinical development are listed in Table I. Similarly, examples of various small molecule inhalation products present in clinical development are listed in Table II [33].

There is also a possibility for vaccination through pulmonary delivery. Back in 1958 already, the possibility of vaccination via inhalation was suspected: a quite small number of living attenuated *Mycobacterium tuberculosis* (fewer than 25 infective units per animal) effectively induced immunity against airborne infection with virulent bacilli in guinea pigs [34].

Another example of this is Spiros® (developed by Dura Pharmaceuticals Inc.), a measles vaccine which is administrable using a DPI. Regrettably, it

Table I *Examples of macromolecules in clinical development (based on [33])*

Macromolecule(s)	Targeted condition
GLP- 1 and insulin	Type 2 diabetes
PTH (parathyroid hormone) and salmon calcitonin	Osteoporosis
Erythropoeitin	Anemia
Interferons	Multiple sclerosis
Growth hormone (GH)	Growth deficiencies
Plasmid complex gene delivery	Hemophilia

Table II *Examples of small molecules in clinical development (based on [33])*

Small molecule	Targeted condition
Dihydroergotamine	Migraine
Fentanyl citrate	Breakthrough pain in cancer
Apomorphine	Male and female sexual diseases
Nicotine	Smoking cessation

was not successfully marketed. One of its weaknesses was that in case of flat batteries, it was prone to failure. Besides, the intricacy of the device led to its expensiveness [1, 35].

A liposomal inhalation formulation has also been developed and FDA-approved for Amikacin, an aminoglycoside antibacterial drug. Arikayce® (ALIS - Amikacin liposome inhalation suspension) is a suspension administered once daily using the Lamira™ Nebulizer System. Amikacin is encapsulated in small liposomes (with a diameter of approximately 300 nm) containing dipalmitoylphosphatidylcholine (DPPC) and cholesterol in a high drug-to-lipid ratio. It is used for the treatment of MAC (*Mycobacterium avium* complex) lung disease in a combination treatment for patients for whom the standard therapy has not been successful and whose substitute therapies are limited or unavailable. It has been shown that ALIS considerably improved the chances of obtaining sputum culture conversion when used as an add-on to guidelines-based therapy (GBT) compared to the use of GBT alone [36, 37].

5.2. Insulin administration through pulmonary delivery

Since the discovery of insulin in 1921-1922 at the University of Toronto [38], there has been a permanent search for a non-injectable yet effective route of administration. In 1987, a study was conducted on six diabetic children where semi-synthetic human insulin was administered via the pulmonary route using a nebulizer. The conclusion was that the blood glucose control gathered was at least as satisfactory as the one obtained following the administration of the regular dose of subcutaneous insulin [39]. Nevertheless, it was acknowledged that inhaled insulin had a considerably reduced bioavailability compared to that of the subcutaneous insulin. Thus, inhaled insulin reached clinical studies only after the delivery devices available, and particle pharmacology reached a certain advancement [40].

The oral and the nasal routes have been the most researched, but none of them ended up in a sustainable product due to the low and inconstant bioavailability. These issues are mostly due to the impermeability of the nasal and gastrointestinal epithelia to insulin. This unsuccessfulness led to the investigation of the pulmonary route since the lungs are naturally permeable to some proteins [41].

Fourteen short studies conducted on both healthy and diabetic patients investigated inhaled regular, soluble insulin as early as in 1925. Additionally, in all studies, a decrease in blood glucose was noted, as well as a considerable absorption of insulin. These events were obtained without the help of penetration enhancers. Moreover, despite early studies apprehensions concerning variable dosing, it has been guaranteed that the inhaled insulin's variability can be equally as good, if not higher than the subcutaneously injected insulin [41].

An example of inhalation insulin was Exubera® (developed by Pfizer Inc.). It was approved by both the FDA (Food and Drug Administration) and the EMEA (European Medicines Agency) for the treatment of type 1 and type 2 diabetes. It was a DPI which operated using blisters of 1 mg or 3 mg of regular human insulin [40]. The greater part of the formulation was insulin (about 60%). The excipients present consisted mainly of a stabilizer (mannitol) [42]. The device consisted of active powder dispersion by pressurized air obtained from a hand piston. Unfortunately, it has not been successfully marketed due to its cost, but also its significant number of operational steps required, and its large size. Insulin requires several administrations, notably in public. Therefore it is highly required for the device used to be convenient and discreet [1, 43]. In 2007, weak sales and poor acceptance of the inhaler prompted Pfizer to discontinue the diabetes drug [6, 40].

A new inhaled insulin example is Afrezza®, an FDA-approved ultra-rapid acting insulin aiming to enhance postprandial glycemic control in diabetic patients, inhaled using a DreamBoat™ inhaler [44]. The administration of Afrezza® is ensured to be secure and effective in type 1 diabetic patients [45].

Table III Deposition mechanisms in the lung (based on [9])

Particle size	Mechanism	Parts of Respiratory tract
Above 5 μm	Inertial impaction	Oropharynx and conducting airways
0.5 – 5 μm	Sedimentation	Bronchi, Bronchioles and Alveoli
0.5 – 3 μm	Sedimentation and Diffusion	
Below 0.5 μm	Diffusion and Brownian motion	Alveolar region

6. Drug deposition and absorption in the lungs

Particle deposition is the first step after inhalation. It can occur in the lungs by three main mechanisms: inertial impaction, sedimentation and diffusion. Other mechanisms are an interception and electrostatic precipitation which are related to the particle shape and the electrostatic charges [9, 46]. The deposition mechanisms in the lungs are demonstrated in Table III.

Inertial impaction is the principle deposition mechanism in the 1-10 generations of the lungs due to the elevated air velocity with the turbulent flow. Larger molecules (above 5 μm) tend to move out with the air stream and impact the wall of the upper airways. The properties of particles, such as density and diameter play a significant role in this case.

Sedimentation is the time-dependent effect on the particles with 0.5-5 μm size in the deeper lung (bronchi, bronchioles and alveolar region) where the velocity decreases. The gravitational force causes the deposition of the particles and is derived by particle diameter, mass, residence time and decreased flow rate. Particles of 3-5 μm reach the tracheobronchial region with sedimentation. *Sedimentation* and *diffusion* are expected in case of particles with a size of 0.5-3 μm which are able to reach the alveolar region.

In the alveolar region, particles with a size smaller than 0.5 μm will deposit according to the *diffusion* principle due to the Brownian motion. Nevertheless, due to their significantly small size, the majority of the particles are exhaled and merely few of them deposit.

Direct *interception* is a phenomenon which usually occurs in case of particles with elongated shape in the upper airways, while charged particles are prone to undergo *electrostatic deposition* [9].

The fate of particles after inhalation depends on the physiology of the patients and the aerosol properties of the particles, namely size, size distribution, shape, charge, density and hygroscopicity [10, 46]. It is well established (Table III) that the size of particles has an important significance in the particle deposition. There are two main calculable parameters which express the aerodynamic properties of aerosols: the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD). MMAD is a diameter which is calculated based on the mass distribution and divides particles into two parts: 50% of the aerosol is larger, the remaining 50% is smaller. If the MMAD is between 0.5 μm and 5 μm, the aerosol is usually considered breathable. The ideal size for a deep lung deposition is 2-3 μm. The GSD demonstrates the variance within the particles, in which the values of 1 and above 1.2 indicate mono-disperse and hetero-disperse systems [10]. Spherical-shaped particles are the most preferred and could easily be manufactured. However, elongated particles are gaining attention due to the lung penetration of fibers. Generally, low-density particles with a large geometric diameter have better aerodynamic properties than high-density particles with a small geometric diameter [10]. There are strategies to alter the aerodynamic diameter (D_{ae}). These are based on the following equation (1), where D_{eq} is the diameter of an equivalent volume sphere of unit density, ϱ_p and ϱ_o are particle and unit densities, and X is the dynamic shape factor [47]:

$$D_{ae} = D_{eq}\sqrt{\left(\frac{\rho_p}{\rho_o X}\right)} \qquad (1)$$

Both large and porous or needle-shaped particles are presumed to have a smaller aerodynamic diameter than their particle size would suggest [47]. All of these parameters aside, the surface charge and the hygroscopicity are also important physicochemical properties. While the surface charge of the cell membrane is negative, small particles with a positive charge can bind easily. However, the mucus layer of the lung epithelium acts as an absorption barrier due to its adhesive surface; therefore it may hinder the penetration of even charged particles [48]. Hygroscopic materials should be formulated carefully in case of DPIs due to the proneness to aggregation [46].

Drug absorption in the lung is also influenced by the physiological environment and clearance mechanisms. In the human lung, the dissolving medium volume is about 10-30 ml with a pH of about 6.6 and consists of 96% water. The remaining 4% are electrolytes and proteins. The drug particles should be in a dissolved state in order to absorb, otherwise they might get cleared up by one of the clearance mechanisms. The principal clearance mechanism is the *mucociliary clearance* (MCC) which is derived by the synchronous sweeping movement of cilia and mucus on the surface of epithelial cells. Their function is the removal of particles from the environment. Therefore, with the propulsive movement of mucus towards the larynx, the particles will be eliminated by swallowing or coughing out, generally within 24 hours. The other possibility is the *alveolar clearance* which is a defence mechanism against harmful microorganisms and particles by macrophages. Particles with a size between 1.5 μm and 3 μm are more susceptible to phagocytosis, and the alveolar permeability is decreasing with increasing molecular weight. There is also a *mechanical clearance* for large particles (above 10 μm) which immediately induces coughing, sneezing or swallowing [9]. The metabolisms in the lungs are similar to the liver. All of phase I metabolizing enzymes can be found in the lung, however, in lower quantities and CYP3A5 is expressed abundantly in the lungs [9].

Figure 9 shows the correlation between the aerodynamic diameter (in μm) and the percentage of deposition in the lungs. The aerodynamic diameter is the diameter of a sphere with the same settling velocity as the particle of interest [50]. As mentioned before, large molecules with an aerodynamic diameter higher than 5 μm tend to impact on the wall of the upper airways and get swallowed. Therefore, the particles with an aerodynamic diameter smaller than 5 μm are the ones with the highest potential for deposition in the lungs. In an aerosol, the ratio of these particles is referred to as either the fine particle dose (FPD) if it is expressed in absolute mass of drug in the particles that are smaller than 5 μm, or the fine particle fraction (FPF):

- The FPD is the mass of particles smaller than 5 μm present within the total emitted dose.
- The FPF is equal to the FPD divided by the total emitted dose.

The higher is the FPF in an aerosol, the higher is the probability of it depositing deeper in the lungs. Consequently, present inhaler devices produce aerosols containing a considerable percentage of particles between 1 and 5 μm [49].

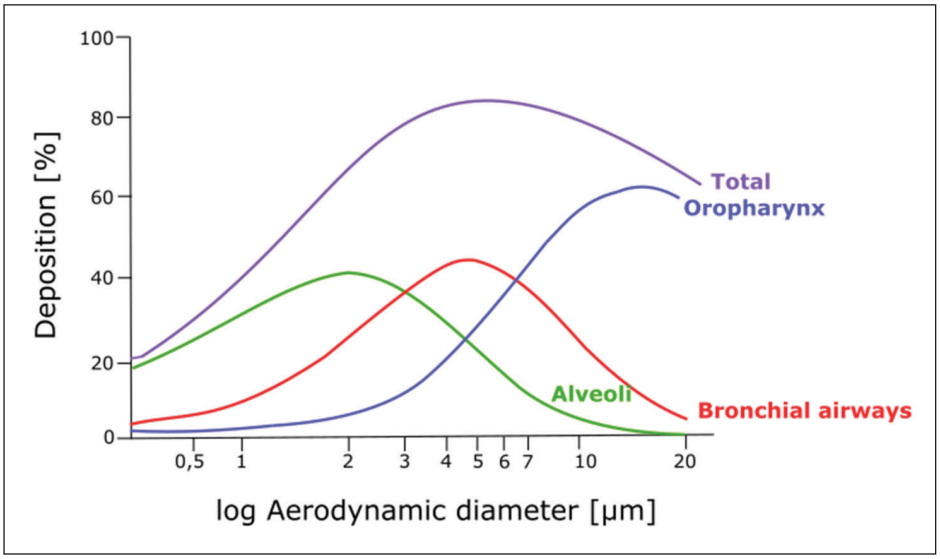

Figure 9 *Relationship between particle aerodynamic diameter and lung deposition (based on [49])*

7. Formulation of inhalation dosage forms

7.1. Excipients in the pulmonary drug delivery

The International Pharmaceutical Excipients Council (IPEC) characterizes excipients as substances in a pharmaceutical formulation other than the active pharmaceutical ingredients which "have been appropriately evaluated for the safety in order to help processing, manufacturing, protection, and give support or to enhance stability, bioavailability, or patient acceptability or to assist in product identification or improve any features of the safety or effectiveness of the drug delivery system during storage or use." [51]. When it comes to pulmonary drug delivery, excipients are particularly necessary in the formulation in order to achieve an optimal size, for instance. As a consequence, excipients for inhaled drugs are usually present in relatively high amounts compared to the active pharmaceutical ingredient.

7.2. Formulations in the pulmonary drug delivery

As seen previously, the inhalation systems can be divided into three main types: nebulizers, pMDIs, and DPIs.

Nebulizers' formulation consists of sterile water combined with co-solvents if needed. It is preferable to keep the number of excipients present in the formulation to a minimum in order to avoid toxicity [9].

The formulation of pMDIs consists of a product concentrate, which will be either solubilized or dispersed in a propellant using an elevated vapour pressure. The formulation itself is quite complex, owing to the presence of both propellants and high vapour pressure [9]. Propellants can be liquefied compressed gas, compressed gas propellants, or chlorofluorocarbons. However, due to their ozone-depleting behaviour, the use of chlorofluorocarbons has been discontinued since January 1st, 1996, following the Montreal Protocol. They since have been replaced by hydrofluoroalkanes [52].

Out of the three main types of inhalation systems, DPIs would be the most advantageous type due to their propellant-free formulation. However, DPIs' formulation requires the use of micronized drug particles. These particles display poor flow properties as well as cohesiveness. Increased Van der Waals, electrostatic forces, and surface tension between the layers of particles result from the high surface area to mass ratio caused by the micronized drug particles [9]. Because of this, their formulation usually requires the active pharmaceutical ingredient to be combined with coarse and fine carrier particles. This will, in turn, ameliorate the flow properties and raise the particle aerodynamic behaviour [53].

The particle size of an aerosol plays a crucial role in delivering the drug to its target site, hence providing an efficient therapy, while simultaneously avoiding clearance mechanisms [54]. In fact, in aerosol therapy, the particle size is believed to be one of the most crucial physical characteristic [30]. In horses for instance, similarly as in humans, particles which are considered to be large (> 10 μm) are either separated and filtered in the upper respiratory tract or collected in the larger airways. Particles which are considered to be medium-sized (6-10 μm) will deposit and accumulate in the larynx, trachea, bronchi, and large diameter bronchioles. Particles which are considered to be small (5 μm or less) will deposit in the small-diameter bronchioles and alveoli; while roughly half of the particles which are considered to be very small (< 1 μm) will deposit in the alveoli, while the other half will be exhaled [30]. Therefore, powder formulations intended for inhalation generally contain micronized drug particles with a particle size between 1 and 5 μm in order to obtain a valuable central and deep lung deposition. These drug particles are usually blended with an inactive excipient of greater size (40μm). Possible inactive excipients are: lactose, mannitol, trehalose, sucrose, sorbitol, and glucose [55]. Among them, lactose (precisely α-lactose monohydrate) is the most frequently used carrier owing to its numerous advantageous properties such as [56]:

- Physico-chemical stability and compatibility (displayed with most low molecular weight drugs)
- Safe toxicological profile
- Availability and affordability

Nonetheless, lactose possesses disadvantages as well: it cannot be administered to patients who are diabetic or lactose-intolerant since it gets ultimately swallowed by the patient, due to its impact on the oropharynx following the device actuation [56]. Due to these reasons, mannitol can be used as an alternative to lactose [57]. It is also relevant to bear in mind that an active pharmaceutical ingredient containing amino groups (proteins, weak bases) cannot be present in the same formulation as reducing sugars because it would lead to instability due to the Maillard reaction [58]. In order to overcome this, non-reducing disaccharides, non-reducing polysaccharides (trehalose and raffinose, for instance), and other sugars are being investigated as carriers [56].

Moreover, several active pharmaceutical ingredients are highly potent: drugs used for the treatment of diseases such as asthma are already effective at a low dose. For instance, only 200-400 μg for salbutamol and 6-12 μg for formoterol are required for the drug to be effective [59]. This arises issues concerning powder handling as well as precise metering of doses. In order to overcome those issues, carriers can be used. The carrier material should meet requirements such as [56, 60]:

- Providing bulk and ensuring flowability
- Decreasing the agglomeration of the particles
- Facilitating powder handling and dosing by providing a larger volume to the formulation
- Helping the dispersion of the micronized drug

Moreover, fine lactose particles present within an equivalent size range as the active pharmaceu-

tical ingredient have been identified as an important element in order to improve the formulation performance [61]. Among the possible explanations for this phenomenon are the presence of active sites and the agglomeration of drug and fine excipients [62]. Inhalation formulations containing a low dose of the active pharmaceutical ingredient tend to be more affected by the properties of the carrier. This might be due to the occurrence of active sites on the carrier as well as their availability [60]. The drug/lactose ratio and the aerosolization performance are correlated, and this correlation is associated with the possible existence of active sites on the surface of the lactose carrier [63].

7.3. Carriers suitable for a small particle size

Powders suitable for inhalation are most commonly obtained by carrier-based systems which consist of two elements: the drug and its carrier.

The drug is usually mixed with larger, coarser lactose particles which act as a carrier and ameliorate the dose reproducibility. As the drug and the carrier are blended, the micronized drug particles attach to the surface of the lactose carrier particles, hence forming an adhesive mixture (Figure 10).

Furthermore, interparticulate forces present in adhesive mixtures are required to be able to produce a blend which is stable and homogenous. Due to the considerable size difference between the drug and its carrier, the unplanned separation of the two should be avoided, while still enabling an easy detachment of one from the other through weak enough drug-carrier interactions. Consequently, the drug delivery occurs through three steps [56]: the detachment of the active pharmaceutical ingredient from its carrier, their dispersion in the airflow, and the deposition in the respiratory tract (Figure 11). In the case of DPIs, the powder formulation is present in the capsule in an aggregated form with a size of 100-150 μm. In order to disaggregate it, a deep and strong inhalation through the device is required, thus reducing it to inhalable particles (1-5 μm) which can successfully deposit in the lungs [64]. This de-aggregation process constitutes a fundamental requirement for DPIs capsules contents [65].

During the drug/carrier detachment in the first step, the fate of the carrier is either to remain in the inhalation device or to deposit in the oropharyngeal region. In adhesive drug mixtures, excipients used as carriers are considered to have a "limited" loading capacity, which makes them convenient for low drug doses. Classic drug-to-carrier ratios are 1:67.5 or 1:99 [56]. The drug quantity which can be processed is limited due to requirements for the content uniformity and stability [66]. The limit is set to 5-10% of the drug, depending on the excipient used in the formulation as a carrier [67].

The use of active pharmaceutical ingredients at a low dose such as dry powders is mainly aimed for the therapy of respiratory diseases (asthma, COPD). In pulmonary drug delivery, the dose of a drug used is considered low when it is in the microgram range (<1 mg) [66]. The drug dose can vary from 6 μg (eformoterol fumarate: Oxis®) to 500 μg (fluticasone propionate: Flixotide®, Seretide®) [68].

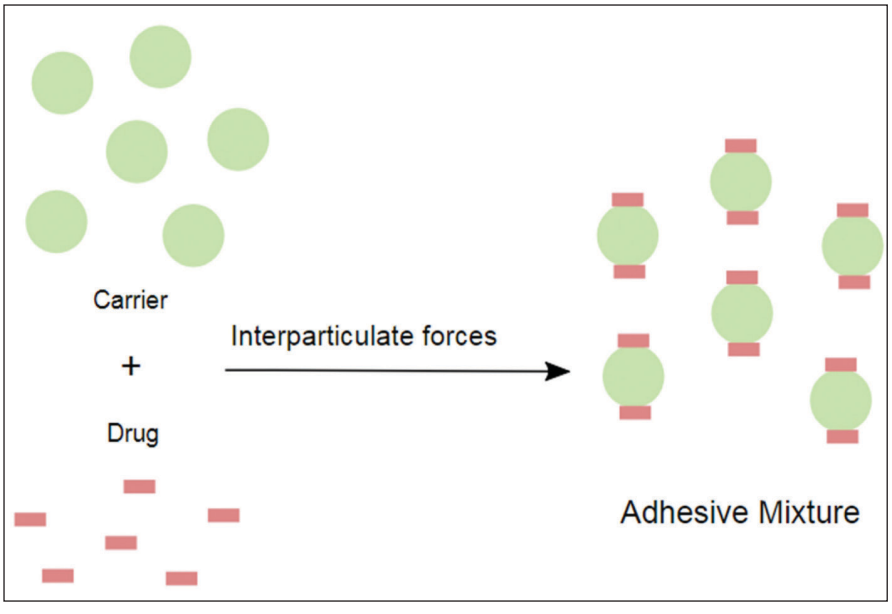

Figure 10 *Adhesive mixture formation*

8. Tests and characterization of aerosols

8.1. Tests

As seen previously, the formulation of inhalation dosage forms plays a great role in their efficiency and is therefore quite elaborated. The processes it involves are complex; thus thorough testing is necessary to ensure the safety and quality of the end product. Table IV displays the gen-

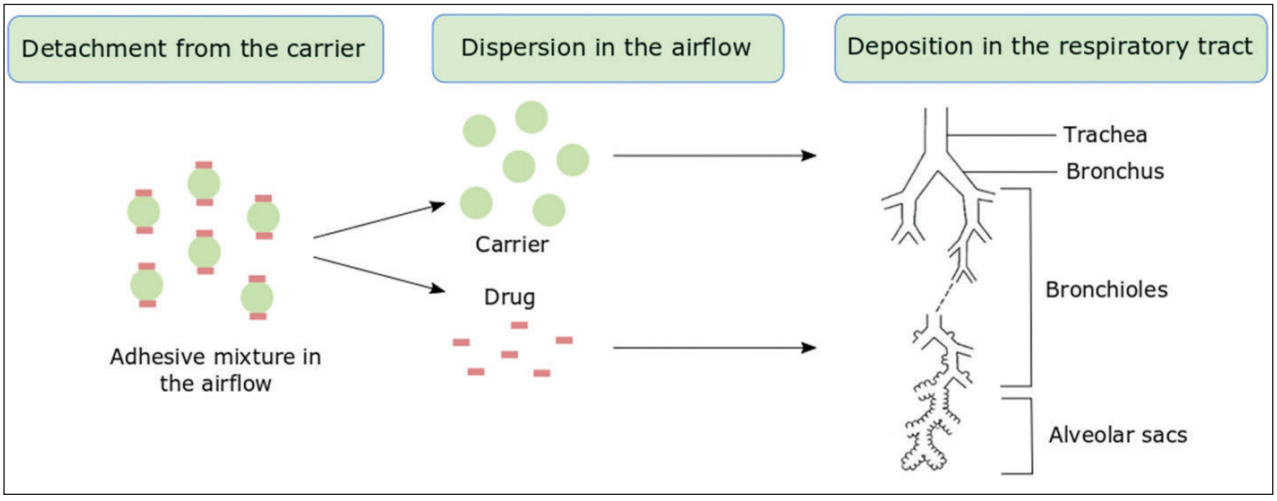

Figure 11 *Illustration of the steps in the drug delivery of carrier-based formulations*

Table IV *Aerosol devices and formulations tests (based on [9, 69])*

Test	Description
Dose uniformity	– Assay of the material or the solvent onto which the dose is sprayed. – Firstly, an empty container is weighed. Secondly, a certain number of doses are sprayed in this same container, which is then weighed again. The difference in weight is then calculated, and divided by the number of doses sprayed.
Particle size determination	The determination is done using a cascade impactor or using a light scattering decay method. The particle size is expressed in μm.
Moisture content	The test can be done by Karl-Fischer or by gas chromatography. The moisture content is expressed in %.
Density	The test can be done using a hydrometer or a pycnometer. A pressure tube is fitted with a Hoke valve, metal fingers, and a hydrometer placed into it. The sample is then put in through the Hoke valve. This causes the hydrometer to elevate in the pressure tube at half height. The density can then be read and is expressed in g/ml.
Vapor pressure	The test can be done by pressure gauge. The pressure variation between containers indicates the presence of air in the headspace. Vapor pressure can also be accurately measured using a can punctuating device. The vapor pressure is expressed as kPa, bar, atm, or psi.
Spray pattern	The aerosol being tested is sprayed on a paper coated with a dye-talc mixture. The dye is either water-soluble or oil-soluble. Particles which collide with the coated paper cause the dye to solubilize and get absorbed onto the paper. This gives a definite spray pattern.
Aerosol valve discharge rate	Two weights are measured: W_1 and W_2. – W_1: Initial weight of a container – W_2: Weight of that same container after being actuated for a particular period of time. The discharge rate with respect to time is obtained by dividing the difference in weights by the time (of the actuation process).
Propellant identification	The identification can be done by gas chromatography or infrared spectrophotometry. These methods can also be used to determine the composition of a blend of propellants. The determination of moisture, halogen, and non-volatile residue provide an acceptability and purity check of the propellants.
Flammability and combustibility (Flash Point)	The test requires a Tag Open Cup (TOC) apparatus. The aerosol product is chilled to -25°F (= -31.67°C) then transferred to the apparatus. The temperature of the liquid is gradually increased. When the liquid's vapor ignites, the "flash point" temperature has been reached. It is expressed in °C.
Flame projection	The test measures the extension of a flame length when the aerosol product is sprayed on an open flame for about 4 seconds. A ruler is used to measure the extension of the flame, and the result is expressed in cm.

eral and additional evaluation tests done on aerosols and their formulation [9, 69].

Characterization of the performance of DPIs can be done using two aspects: the patient's inspiratory flow and the turbulence generated inside the inhaler itself. Since DPIs are breath-actuated devices, the powder formulation they deliver requires the patient to supply turbulent inspiratory forces to break down the powder aggregates into fine particles of less than 5 μm in diameter in order for them to deposit deeper in the lungs. As mentioned before, optimal use of the dry powder inhaling device is determined by the patient providing an appropriate inspiratory flow, and the turbulence generated by the intrinsic resistance of the inhaler, which is influenced by the design of the device. Basically, three varieties of designs are available, with a low (such as the Breezhaler®), medium (such as the Ellipta®), or high resistance (such as the Handihaler®). The duplicability of the dose by the device at different flow rates demonstrates how the incorrect manipulation of the inhaler affects the system. The higher the inspiratory flow, the better the performance of the device since the drug dose inhaled by the patient is increased [70, 71]. A favourable peak inspiratory flow rate should be of minimum 60 L/min through the inhaler to achieve disaggregation of the powder formulation. The device-specific airflow resistance is determined using the pressure drop and the flow rate following Ohm's law, as shown in equation (2) [72]. Higher airflow resistance is associated with more considerable difficulties for patients (particularly in children and elderly people) to adequately operate the device with an appropriate flow rate, which makes the airflow resistant a significant parameter [70].

$$R = \frac{\Delta P}{Q} \qquad (2)$$

where R = Resistance, ΔP = Pressure drop, Q = Flow rate.

Oxis Turbuhaler® and Foradil Aerolizer® DPIs have been tested *in vitro* to study their efficiency in delivering 12 μg of Formoterol fumarate, a β_2-sympathomimetic drug, and 600 μg of lactose monohydrate. Four flow rates were investigated (28.3, 40, 60, and 80 L/min). The optimum flow rate for both inhalers ranges between 40 and 60 L/min to deliver a satisfactory bronchodilation effect. The study concluded that Oxis Turbuhaler® delivered smaller particles; thus a deeper lung deposition was achieved. However, the high specific airflow resistance is a factor which will affect the usage of the device by severe asthmatic children. Nevertheless, no difference in their bronchodilating effect was found [73].

8.2. Particle size determination

8.2.1. Cascade Impactor

The determination of the therapeutic activity of an inhalation aerosol is dependent on the particle size [69]. Cascade Impactors are mentioned in the Pharmacopeia and therefore constitute the most commonly used devices for the *in vitro* study and measurement of the particle size distribution of inhaled formulations. This is mainly due to their numerous advantages such as the direct measurement of aerodynamic particle size, and the determination of drug mass in the formulation with different size ranges, without the disturbances from the excipients [17].

Originally, the Andersen Cascade Impactor (ACI) was the most commonly used impactor in the pharmaceutical industry. However, the ACI displayed certain disadvantages and has since been replaced by the Next Generation Impactor (NGI), specially designed for inhaler testing [74] (Figure 12).

Cascade impactors rearrange particles and droplets in an aerosol based on their aerodynamic diameter. They use particle/droplet inertia impaction to segregate particles and droplets from a moving airstream [69]. The NGI comprises 7 stages (Figure 13), 5 of which are in a 0.5-5 microns range. Each stage contains a set of nozzles. The airflow moves

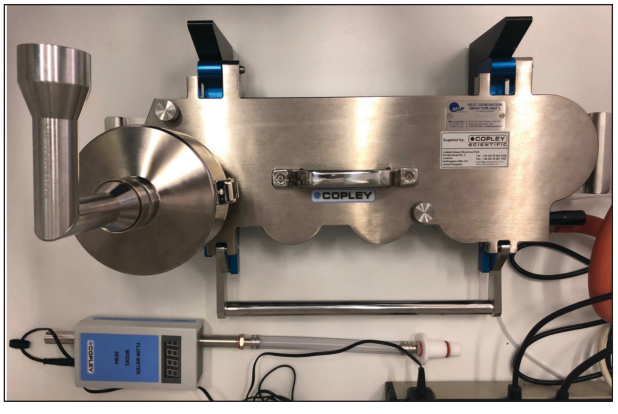

Figure 12 *Next Generation Impactor (NGI) with induction port and preseparator*

across the apparatus following a sawtooth pattern. The velocity of the airstream is increased as it is forced to cross through a sequence of nozzles comprising progressively decreasing jet diameters. This results in particle separation as well as sizing [74].

Another particular feature of the NGI is also its micro-orifice collector (MOC). It catches highly small particles in a collection cup, which can be evaluated along with the particles collected in the stages cups [75]. Aerodynamic properties can be calculated from the collected data.

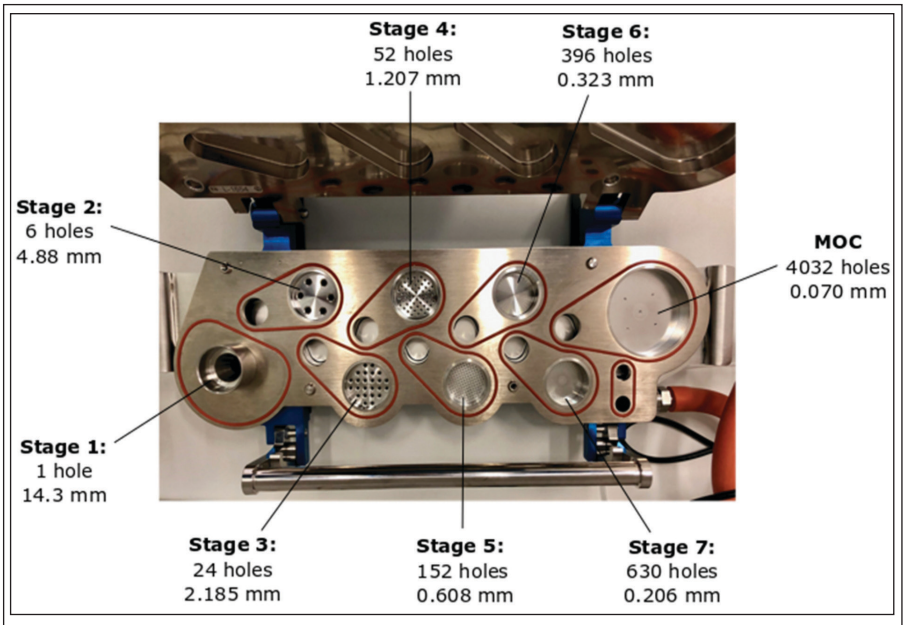

Figure 13 *NGI stages and micro-orifice collector (MOC) with their number of holes and nozzle diameter (based on [75, 76])*

Figure 14 shows the Spiriva HandiHaler® placed on the induction port of the NGI. HandiHaler is an 18 μm/capsule DPI made by Boehringer Ingelheim/Pfizer Inc. Its powder is delivered at a flow of 20-30 L/min, making it a flow-limiting device [77, 78].

Figure 15 demonstrates the Serevent Evohaler® placed on the induction port of the NGI. Serevent Evohaler is a 25 μm/actuation inhaler made by GlaxoSmithKline Inc. It is a CFC-free pMDI [79].

Figure 14 *HandiHaler placed on the induction port of the NGI*

8.2.2. Laser Diffraction

Particle size analysis can also be performed using optical methods as an alternative, such as laser diffraction (light scattering), laser Doppler, or time-of-flight. The most widely used among these is the laser diffraction [17]. The Cascade Impactor analysis can be substituted by laser diffraction in order to assess the particle size, however, aerodynamic diameters cannot be measured with this technique. Moreover, the main advantage offered by NGIs is the determination of the FPF and other size fractions. The other methods, such as laser diffraction provide no differentiation. It simply measures the overall particle size distribution in the sample [80]. However, laser diffraction is considered to be a remarkably accurate, flow rate-independent and a rather quick method and therefore constitutes an excellent alternative for the cascade impactor measurements. This technique has been used since the 1980s to measure the particle size of nebulized drug solutions [81].

The laser produces a monochromatic, coherent, parallel beam which is widened by the beam expander unit (Figure 16). This beam then casts a light on the dispersed particles. The light produced is scattered by the dispersed particles. These particles in the measuring zone form scattering patterns and these patterns carry information on the number of particles, the particle size, and the particle shape in any orientation [82, 83]. Examples of laser diffraction instruments are:

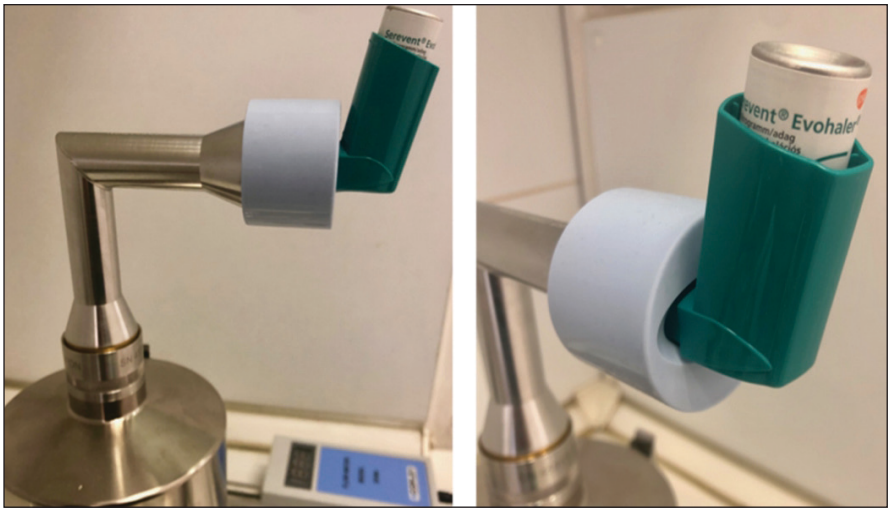

Figure 15 *Evohaler placed on the induction port of the NGI*

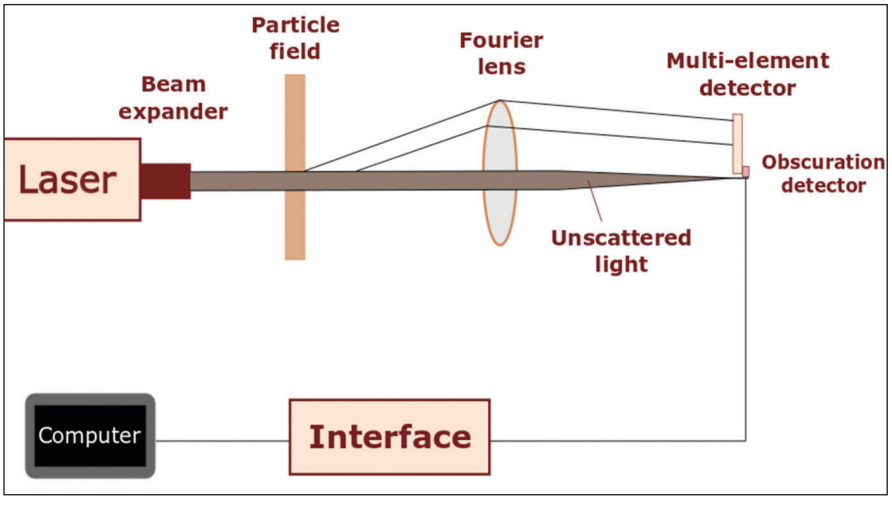

Figure 16 *Laser diffraction particle size analyzer layout (based on [82])*

- RODOS for dry dispersions
- HELOS (Helium-Neon Laser Optical System) laser diffraction sensor which can be connected to different inhalers (DPI, MDI, Soft mist inhalers)
- MYTOS which makes possible the in-line dry particle size analysis for automated laboratories

All are developed by Sympatec Inc. They provide a precise particle size analysis for dry powders in the size range below 0.1 μm to 4000 μm for RODOS, 0.1 μm to 8750 μm for HELOS, and from 0.25 μm to 3500 μm for MYTOS [84].

In the case of dry powders, sampling before measuring is a significant step. An ineffective sampling technique will result in a wrong characterization of the powder. In laser diffraction, powder measurements can be carried out by dry dispersion, which aim is to enable the measurement of particles individually. This is done through the separation of particles agglomerates by suppressing the forces which bind these particles together. This can be achieved using the RODOS injection disperser (Sympatec Inc.). With RODOS, the compressed air injector provides an effective, long, and straight dispersion line of dry solid particles with a special flow control which can mix the particles with inert gas or compressed air. This high airflow, the jet formation and expansion provide an optimal aerosol cloud for the optical analysis without aggregation. Measurements directly from the aerosol cloud are also possible using systems such as HELOS (Sympatec Inc.), which provides a distribution analysis for powders and granules, but also wet samples such as suspensions, emulsions, and sprays [80, 84].

However, this technique possesses drawbacks as well: the measuring zone lacks a suitable air extraction which would be required in order to prevent the aerosol droplets from entering back into the laser beam. Air suction is also necessary to analyze how the inspiratory flow rate affects the droplet size distribution. This shortage is explained by the fact that a junction is needed between the nebulizer's mouthpiece and the vacuum system in order to obtain a controlled air extraction. Nevertheless, it is not easily achievable and rather complicated to do so due to interferences with the laser beam. Other limitations include [80]:

- The calculation of volume distribution curves presuming that the particles possess a spherical shape.
- The inability to measure aerodynamic diameters (geometric particle diameters are measured instead).
- The inability to acquire fine particle mass fractions.
- The inability to measure low particle concentrations (dose weights < 4 μg).
- The inability to control the flow curve through the inhalation device.

- Mixtures measurements (drug-drug or drug-excipients) are rather difficult.
- The measuring range is broader than the drug particles' size distribution: a 0.9 μm – 175 μm range for a 100 mm lens, and a 0.45 μm – 87.5 μm range for a 50 mm lens.

Nonetheless, laser diffraction remains a technique with several advantages, such as numerous size classes, precision, and a brief measuring time.

8.3. Dissolution

The study of adissolution behaviour is the study of a solid substance's potential to penetrate a solvent medium based on affinity characteristics. This provides useful information on the substance's absorption behaviour *in vivo* [17].

The dissolution profile of a dosage form constitutes an essential parameter for the bioavailability of any drug that is applied by a non-parenteral route. Factors that influence the dissolution behaviour of a drug include drug solubility, drug dose, formulation properties, drug particle properties, and the epithelial lining fluid (ELF) composition (which changes along the respiratory tract) [85]. In the lungs, the dissolution is the most significant in the small bronchioles and the alveoli, which constitute the segment where the majority of the drug absorption takes place. After inhalation, the fate of the drug particles is to dissolve in the ELF present along the respiratory tract. The ELF consists of a surfactant layer and an aqueous phase [85]. According to the region of the lungs where it is situated, the lining fluid differs in composition, thickness, and volume: the trachea, bronchi, and bronchioles are enveloped with a thick mucus gel (approximately 3 – 23 μm) whereas the alveolar region is covered with a particularly thin film (approximately 0.07 μm). The progressing thinning of the ELF results in physiological dissimilarities, which in turn induce difficulties in establishing the residence time of the particles by simulating lung conditions [86]. After inhalation, the particles which penetrate the non-ciliated segment of the pulmonary region will dissolve in the ELF. Only this dissolved portion of the dose administered will be accessible for absorption through the alveolar membrane [17].

Currently, the pharmacopoeia does not list any standard method for the dissolution testing of inhalation drugs despite the fact that several methods have been designed [17, 86, 87]. A certain number of methods are available to study conventional solid dosage forms, but they are intended for the simulation of the gastrointestinal tract and are therefore not suitable to study the dissolution of inhaled drugs (mainly due to their "sink" conditions") [17]. An example of these methods is the paddle over disc dissolution setup. This method enables the evaluation of the *in vitro* dissolution rate of inhaled formulations [88]. Another method is the flow-through cell apparatus, which studies the dissolution profile of poorly soluble glucocorticoids aerosols [89]. The dissolution profile of inhaled formulations can also be assessed using the diffusion-Franz cell apparatus. A study evaluating these three methods (the paddle over disc method, the flow through cell, and the diffusion-Franz cell apparatus) concluded that the Franz cell apparatus was the most promising [17, 90]. The Franz cell apparatus consists of a donor and receptor compartment. These two compartments are separated by a semi-permeable cellulose membrane. The powdered formulation is set on the bottom surface of a filter membrane, which is then positioned on the semi-permeable cellulose membrane. The drug will then dissolve and diffuse through the semi-permeable membrane. The study is done on samples gathered from the receptor compartment. The dissolved drug which diffuses through the

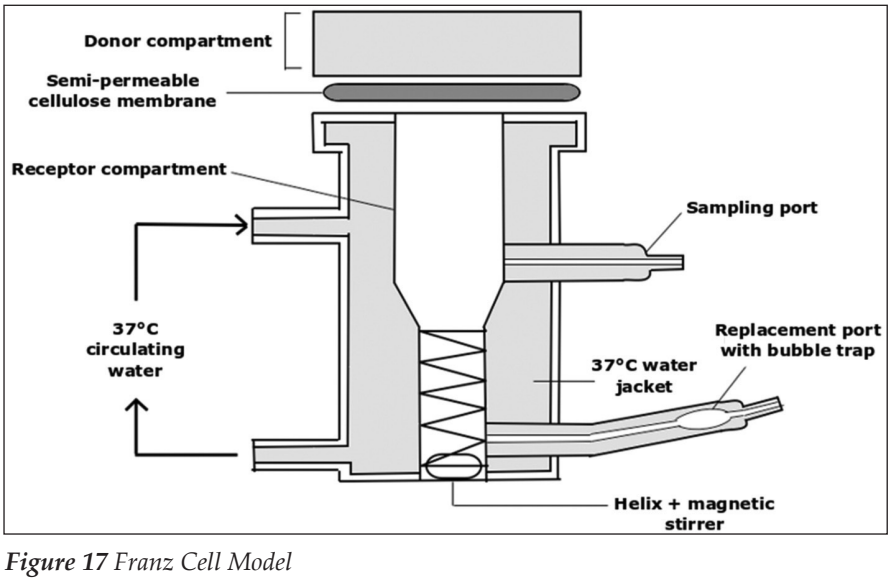

Figure 17 *Franz Cell Model*

membrane is analyzed by collecting the samples from the acceptor compartment (Figure 17).

Moreover, developing a method for the dissolution testing of an inhaled drug applicable to the lungs and their physiology can be quite complex due to the lungs' characteristics such as the small amount of their fluid. Besides, a separation of the API from the excipients should be done before testing [17]. However, a recent study developed an apparatus aiming to investigate the dissolution behaviour of anti-tubercular drugs powder formulations. This apparatus consists of a flow perfusion cell which symbolizes the air/blood perfusion. It employs a small volume of 25 μL of unstirred, stationary medium to study the *in vitro* dissolution of inhalable drug particles, notably moxifloxacin and ethionamide, two anti-tubercular drugs. The particles of these two drugs were set on a sheet glass with the help of a Twin Stage Impinger. The mucus in which they should dissolve was simulated by polyethylene oxide (PEO) in a phosphate-buffered saline (PBS). Their dissolution profile was then investigated. The study concluded that ethionamide has a lower solubility and displayed a slower dissolution rate than moxifloxacin under all the dissolution conditions applied. The dissolution behaviour of ethionamide brought into question the dissolution mechanisms in small volumes of stationary medium (simulating the *in vivo* tracheobronchial region of the lungs). Such questions could be addressed using this method [17, 86, 88].

The tracheobronchial region of the respiratory tract is covered with a viscoelastic blend containing glycoproteins, proteins, and lipids. However, the composition of this mucus may vary in case of diseases [91]. In testing conditions, an accurate simulation of *in vivo* parameters is continuously required. In dissolution testing, it can be achieved through the use of biological simulated lung fluids (SLF), which serve as dissolution media.

The SLF was first developed by Moss in 1979 [92]. This simulated fluid provides a greater comprehension of *in vivo* mechanisms. However, as seen previously, the development of a standardized dissolution method is a complex project due to the special characteristics of the lungs, such as the particularly small volume of fluid present.

Despite that, research was done to study a possible reproducible, standardized test method to investigate the dissolution profile of a micronized hydrocortisone pulmonary formulation. This method includes a dissolution test station (with mini-paddles, dissolution vessels, a water bath, and a sampling probe) and a membrane cassette. Among other things, the influence of the SLF composition on the dissolution rate was investigated. A modified version of the SLF (mSLF) was also used, which consists of 100 ml of SLF + 200 μg/mL of 0.02% (w/v) of dipalmitoylphosphatidylcholine (DPPC). The samples of hydrocortisone in SLF and mSLF were examined using an HPLC system. The mSLF yielded an increase in the release rate of hydrocortisone because a DPPC-containing medium may enhance the wettability of hydrophobic drugs and inhibit the aggregation of particles, therefore increasing the dissolution rate [88].

Another study designed a biorelevant SLF with distinct composition and characteristics (Table V), as well as directions for usage and storage [93].

Conclusion

Overall, it has been established that the pulmonary drug delivery demonstrates a significant relevance, notably due to the physiology of the lungs and their advantageous properties. Despite the presence of some disadvantages such as clearance mechanisms, pulmonary drug delivery continues to be a non-invasive route of administration that is preferred by patients. It has also been shown that pulmonary delivery is one of the oldest drug delivery methods. Since its first use, its application has gotten more and more elaborated. Nowadays, several types of inhalation devices are available on the market: nebulizers, MDIs, and DPIs. Supplementary devices such as spacers are also employable in order to achieve optimal results. The performance

Table V *Composition and characteristics of a simulated lung fluid (based on [93])*

Composition	Physicochemical parameters
Dipalmitoylphosphatidylcholine (DPPC) Dipalmitoylphosphatidylglycerol (DPPG) Cholesterol Albumin IgG Transferrin Antioxidants	pH=7.2 Viscosity = 1.138 x Pa.s Conductivity = 14.5 ms Surface tension = 54.9 mN/m Density = 0.999 g/mL

and effectiveness of such inhalation devices depend partly on the particle size of the formulation used, as well as its deposition in the lungs. Such deposition is achieved by several mechanisms: diffusion, sedimentation, impaction, and interception. Furthermore, in order to assure the highest possible efficiency, formulations are developed following several criteria. Therefore, thorough testing is required to ensure the safety and quality of the end product. These tests are performed on both the formulation and the device which carries it. Investigations of the particle size and aerodynamic properties, for instance, can be done using laser diffraction and a cascade impactor.

Summing it up, the pulmonary drug delivery constitutes a considerably advantageous route of administration for local and systemic treatments of diseases. Nevertheless, the development of pulmonary dosage forms is a complex process which requires extensive considerations and meticulous optimizing.

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Epidemiology and species distribution of anaerobic Gram-negative cocci: a 10-year retrospective survey (2008-2017)

MÁRIÓ GAJDÁCS[1,2*], EDIT URBÁN[3]

[1]*Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, 6720 Szeged, Eötvös utca 6., Hungary*

[2]*Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged, 6725 Szeged, Semmelweis utca 6., Hungary*

[3]*Department of Public Health, Faculty of Medicine, University of Szeged, 6720 Szeged, Dóm tér 10., Hungary*

**Corresponding author: Márió Gajdács*
gajdacs.mario@pharm.u-szeged.hu

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Abstract

Introduction: *The group of anaerobic Gram-negative cocci (AGNC) includes the genera Veillonella, Megasphaera, Anaeroglobus, Negativicoccus and Acidaminococcus. These bacteria are an integral part of the microbiome of humans but may be causative agents in various infectious processes. The available data on the epidemiology and significance of AGNCs is scarce.*

Aims: *To assess and compare the prevalence of different species of AGNCs among inpatients and outpatients at the Albert Szent-Györgyi Clinical Center retrospectively, during a 10-year study period.*

Methods: *Isolates containing AGNC were identified retrospectively by reviewing the online microbiology records of the Institute of Clinical Microbiology.*

Results: *The median age of affected patients overall was 52 years (range: 1–90 years), with a male dominance. 59.79% of samples originated from inpatients. 572 individual AGNCs isolates were recovered from clinical samples, most of the isolated GNACs were Veillonella spp. (95.28%), Megasphaera and Acidaminococcus species accounted for a minority of isolates (2.79% and 1.93%, respectively), while Anaeroglobus and Negativicoccus species were not isolated. In the second half of the study period (2013-2017), 91.31% of isolates were identified on the species level ($p<0.001$) using MALDI-TOF MS.*

Conclusion: *The current study represents a long-term surveillance study on the isolation frequency and trends among anaerobic Gram-negative cocci (AGNCs), isolated in the Southern Great Plain of Hungary, highlighting the beneficial effect of MALDI-TOF MS on the diagnostic efficacy of the laboratories*

Keywords: *anaerobic bacteria, Veillonella spp., MALDI-TOF MS, clinical microbiology, epidemiology*

1. Introduction

The group of anaerobic Gram-negative cocci (AGNC) includes the genera *Veillonella*, *Megasphaera*, *Anaeroglobus*, *Negativicoccus* and *Acidaminococcus* [1]. These bacteria (with *Veillonella* species in the highest numbers among the members of the group) are an integral part of the microbiome of the oral cavity and the gastrointestinal, genitourinary and respiratory tracts of humans [1,2]. Before the advent of molecular methods (polymerase chain reaction) and novel diagnostic techniques in the clinical microbiology laboratories (e.g., mass spectrometry, sequencing), identification of anaerobic Gram-negative cocci was based solely on Gram stain morphology and anaerobic cultivation [2-5]. Awareness regarding the roles of anaerobes in various infectious diseases has increased in recent years, especially due to the pronounced interest towards the study of the human microbiome; although the organisms mostly studied were *Clostridium*, *Bacteroides*, *Prevotella*, *Fusobacterium* species and Gram-positive anaerobic cocci (GPAC) [6-8]. There is a distinct lack of data and an uncertainty regarding the prevalence and importance of AGNC species in human infections: these organisms are recovered in pure culture relatively infrequently, in addition, many laboratories do not have the suitable resources and equipment (e.g., GasPack jars, anaerobic glove boxes) for their isolation and identification [9,10]. The epidemiology of different pathogens shows great variation among time periods and geographic regions therefore, the assessment of local data is essential to evaluate trends over time and to reflect on the national situation, compared to international data. The aim of this study was to assess and compare the prevalence of different species of AGNCs among inpatients and outpatients at the Albert Szent-Györgyi Clinical Center (Szeged, Hungary) retrospectively, during a 10-year study period.

2. Materials and methods

2.1. Study design

The Albert Szent-Györgyi Clinical Center is an 1,820-bed (1,465 active and 355 chronic beds, respectively), primary and tertiary care university-affiliated (University of Szeged) teaching hospital, servicing an urban and rural population in the southeast region of Hungary of about 405,000 people.

Isolates containing AGNC were identified retrospectively by reviewing the online microbiology records of the Institute of Clinical Microbiology. The data screening included samples taken at inpatient departments and outpatient clinics over a 10-year period (January 2008–December 2017). In addition, patient data was also collected, limited to demographic characteristics (age, sex, inpatient/outpatient status) and sample type. Isolates were considered separate if they occurred more than 30 days apart or different AGNCs were isolated [11,12].

2.2. Identification of isolates

The processing of samples arriving to the Institute of Clinical Microbiology was carried out according to guidelines in routine clinical bacteriology. The cultivation of anaerobic bacteria was carried out in line with principles in anaerobic bacteriology, at 37°C in an anaerobic chamber (Baker Ruskinn, York, UK). Between 2008 and 2012, the identification of anaerobic isolates was carried out based the presumptive methods recommended by the Wadsworth Anaerobic Bacteriology Manual, additionally, with the Rapid ID 32A (bioMérieux, Marcy-l'Étoile, France) identification kit [13]. From 2013 onwards, identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Bacterial cells were transferred to a stainless-steel target using a sterile toothpick. An on-target extraction was performed by adding 1 µl of 70% formic acid prior to the matrix. After drying at ambient temperature, the cells were covered with 1 µl matrix HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid). Mass spectrometry measurements were performed by the Microflex MALDI Biotyper (Bruker Daltonics, Bremen, Germany) in positive linear mode across the m/z range of 2 to 20 kDa; for each spectrum, 240 laser shots at 60 Hz in groups of 40 shots per sampling area were collected [5]. The MALDI Biotyper RTC 3.1 software (Bruker Daltonics, Bremen, Germany) and the MALDI Biotyper Library 3.1 were used for spectrum analysis. Antibiotic susceptibility testing is not routinely performed from AGNC isolates [14,15].

2.3. Statistical analyses

Descriptive statistical analysis (including means or medians with ranges and percentages to characterize data) was performed using Microsoft Excel 2013. Additional statistical analyses were performed with SPSS software version 24 (IBM SPSS Statistics for Windows 24.0, Armonk, NY, IBM Corp.), using the χ^2-test and Mann-Whitney's U-test to compare groups. P values below 0.05 were considered statistically significant.

3. Results and discussion

3.1. Study population

The median age of affected patients overall was 52 years (range: 1–90 years), with a male dominance (56.64%, with male-to-female ratio of 1.31), which was present is most of the individual study years (Table I). 59.79% of samples originated from inpa-

Table I *General characteristics of the study population (grouped by study year)*

Study year	2008	2009	2010	2011	2012
No. of affected patients	19	29	27	21	39
Male-to-female ratio	2.17	1.23	0.80	1.33	0.74
Percentage of inpatients	57.89%	68.97%	48.15%	61.90%	58.97%
Median age [yrs]	55	50	47	56	52
Age range [yrs]	(1-75)	(6-87)	(3-79)	(18-90)	(2-84)
Study year	2013	2014	2015	2016	2017
No. of affected patients	30	44	68	134	159
Male-to-female ratio	1.00	2.33	1.34	0.56	1.26
Percentage of inpatients	63.33%	65.00%	69.12%	63.43%	52.20%
Median age [yrs]	54	55	55	49	51
Age range [yrs]	(11-83)	(4-84)	(1-86)	(8-81)	(7-83)

Table II *Origin of samples of interest at the Albert Szent-Györgyi Clinical Center (SZTE)*

Department/Clinic	%
ENT	29.29
Surgery	16.07
Internal Medicine	11.61
Oral Surgery	10.89
Neurology	0.71
Obstetrics and Gynecology	3.21
Pediatrics	3.93
Dermatology	8.93
Intensive care	5.71
Emergency	1.61
Ophtalmology	0.36
Traumatology	5.71
Urology	1.96

ENT: Dept. of Otorhinolaryngology

tients. No statistically significant difference was observed between the patient characteristics (gender, age, inpatient/outpatient status; p>0.05), however, a slight dominance of patients ≥50 years of age could be noted in both the inpatient and outpatient groups (58.48% for inpatients and 63.63% for outpatients, respectively).

Almost one-third of the samples originated from the Department of Otorhinolaryngology (29.29%); other major contributors to positive samples include the Departments of Surgery (16.07%), Internal Medicine 11.61%) and Oral Surgery (10.89%), while the remaining 32.14% of samples originated from nine other institutions (Table II).

31.45% of isolated GNACs originated from abscess samples, while various wound isolates obtained by invasive sampling methods (19.82%) or superficial swabbing (15.64%) were also prevalent. Other positive samples include bile specimens, puncture samples from the sinuses and inner ear, bronchoalveolar lavage, blood cultures and intrauterine devices (IUD) (33.09%). The composition of sample types that were positive for AGNCs is not surprising as most of these originated from anatomical regions, where these bacteria as present as normal flora, in addition to being relevant pathogens, if there is an underlying pathology or surgical intervention in the patient's anamnestic data.

During the 10-year study period, 572 individual AGNCs isolates were recovered from clinical samples at the Albert Szent-Györgyi Clinical Center. Most of the isolated GNACs were *Veillonella* spp. (95.28%), including *V. parvula* and *V. atypica* (corresponding to more than half of the isolated species) as the most frequently isolated genera during the study period; in contrast, *Megasphaera* and *Acidaminococcus* species accounted for only a minority of isolates (2.79% and 1.93%, respectively), while *Anaeroglobus* and *Negativicoccus* species were not isolated (Table III). AGNCs were isolated as standalone pathogens in only 32.87% of cases, they were mostly isolated together with other bacteria, as part of a complex aerobic/anaerobic flora. The number of isolated AGNCs was significantly higher in the period after 2013 (136 vs. 436 isolates; p<0.001). In the first half of the study period (2008-2012), most of the isolated pathogens were identified only at a *genus* level (78.83%), while in the second half of the study period (2013-2017), 91.31% of isolates were identified on the *species* level (p<0.001).

4. Conclusions

The current study represents a long-term surveillance study on the isolation frequency and trends among anaerobic Gram-negative cocci (AGNCs), isolated in the Southern Great Plain of Hungary over a 10-year period. To the best of our knowledge, this is the first and longest-spanning study reporting on their prevalence (and infections caused by these pathogens by proxy) in Hungary.

Based on the results of this retrospective survey, the most prevalent isolates at our tertiary-care center were *Veillonella* spp. (which may be considered the anaerobic counterparts of *Neisseria* species), while other species in the group only represented a fraction of the isolated species; this is in line with the findings of the few studies available in the literature. Hospitalization, a slight male dominance and the age (>50 years) of many affected patients also correspond to literature findings [1-3,5,10]. There was a significant increase in both the number of isolated AGNC species and their correct (species-level) identification in the second half of the study period. Both may be explained by the introduction of the MALDI-TOF MS identification system to the diagnostic workflow of the laborato-

Table III *Species distribution among GNACs in the study period (2008-2017)*

***Veillonella* spp.**	**545**	**(95.28%)**
V. parvula	238	(41.60%)
V. atypica	103	(18.01%)
V. dispar	56	(9.79%)
V. denticariosi	5	(0.87%)
V. ratti	2	(0.35%)
***Megasphaera* spp.**	**16**	**(2.79%)**
Megasphera muciniformis	10	(1.75%)
***Acidaminococcus* spp.**	**11**	**(1.93%)**
Acidaminococcus intestinum	8	(1.39%)

ry. Several studies have reported on the beneficial changed MALDI-TOF put forth in clinical microbiology, both in regard to the speed and the quality of identification [16]. This is especially true with anaerobic bacteria, as their cultivation and identification with conventional methods is time- and resource-consuming [5,9].

If we analyse these results from a clinical perspective, this would mean that 1-2 positive samples were received by the Institute of Clinical Microbiology per week, where significant growth of AGNCs was detected. In these cases, the continuous communication between physicians and the diagnostic microbiology laboratory is crucial. The role of the laboratory is to supply clinically relevant information in a precise and timely manner, which should be reciprocated by the feedback of the physicians, beginning from the submission of the sample, followed by information regarding the symptoms of the patient and the clinical picture [9].

Some limitations of this study must be acknowledged. Firstly, the presence and nature of symptoms of the patients are unknown; additionally, due to the inability to access the medical records of the individual patients affected, the correlation between the existence of relevant underlying illnesses (e.g., Type 2 diabetes, surgical interventions, iatrogenic or disease-related immunosuppression) and the infection could not be assessed. Furthermore, antimicrobial susceptibility testing of the isolated AGNC species was not performed, therefore no information is presented regarding the resistance trends in the isolated bacterial strains.

5. Acknowledgements

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6. Competing interests

The authors declare no conflict of interest, monetary or otherwise.

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Solubility analysis of venlafaxine hydrochloride polymorphs by shake-flask method and real time monitoring

KRISZTINA TAKÁCS-NOVÁK[1*], DÓRA TEMPFLI[1], DÓRA CSICSÁK[1], GERGELY VÖLGYI[1], ENIKŐ BORBÁS[2], ZSOMBOR K. NAGY[2], BÁLINT SINKÓ[3]

[1]*Department of Pharmaceutical Chemistry, Semmelweis University, H-1092 Budapest, Hőgyes Endre u. 9., Hungary*
[2]*Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, H-1111, Budapest, Műegyetem rakpart 3., Hungary*
[3]*Pion Inc. Billerica, Massachusetts 01821, United States of America*

**Corresponding author: Krisztina Takács-Novák*
Email: novak.krisztina@pharma.semmelweis-univ.hu

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Abstract
Aims: *The aqueous solubility of two polymorphic forms of venlafaxine hydrochloride was investigated.*
Methods: *The pH-dependent solubility (S_{pH}) over a wide pH range was measured by saturation shake-flask (SSF) method at 25 °C. The solubility of the free base form was depicted by the intrinsic solubility (S_o). To identify the solid form present at the solubility equilibrium, X-ray powder diffraction (XRPD) and Raman spectroscopy was carried out. The dissolution was studied using real time concentration monitoring applying fiber optic UV probes.*
Results: *No difference was found in the S_{pH} values of Form I and Form II, in the pH range 7.5-12. Solid phase isolated from pH 10-12 suspensions was identified as free base by XRPD and Raman spectroscopy. Precipitates separated from pH 7-8 samples were also identical product. The transition of polymorphs to the free base was supported by the real time dissolution analysis.*
Conclusion: *In this study we demonstrated a good agreement of equilibrium solubility measured by SSF method and in-situ UV fiber optic method. µDISS Profiler™ has the advantage to provide much more information about dissolution process; with this approach the dissolution kinetic, the supersaturation and the time needed to reach the equilibrium can be easily monitored.*

Keywords*: solubility-pH profile, polymorphs, shake-flask method, µDISS Profiler, venlafaxine*

1. Introduction

Solubility is one of the most important molecular properties of drugs. It plays a key role in determining their absorption potential and has fundamental impact on the bioavailability therefore. Solubility data refers to the given physical form of a solid compound thereby it is significantly affected by polymorphism. Polymorphism – the ability of a substance to exist as two or more crystalline forms that have different arrangements and/or conformations of the molecules in the crystal lattice – is common among the pharmaceutical solids [1]. More than one third of drugs show polymorphism and a further one third is capable of forming hydrates and solvates [2]. This subject has gained intensive attention in both original and generic drug research since in case of several drugs polymorphism caused serious safety problems [3]. The most known examples (from chloramphenicol palmitate, through carbamazepine, oxytetracycline, enalapril, etc. up to ritonavir) have been extensively reported in the literature [4-8]. These cases induced severe regulatory considerations [9] resulting that polymorph screening and the investigation of physico-chemical properties and stability of different polymorphic forms has become inevitable during the process of drug development [10, 11].

Polymorphs have different lattice energy so they differ in those physical and chemical properties which associated with the internal free energy of the solid, such as: density, refractivity, conductivity, hygroscopicity (packing properties); melting temperature, vapor pressure, solubility (thermodynamic properties); dissolution rate, stability (kinetic properties); and several others (surface, mechanical, spectroscopic properties) [2]. From absorption and bioavailability point of view the solubility and the dissolution rate are the most relevant properties of a pharmaceutical compound, however from formulation and safety aspects stability is also essential. A general rule is that a polymorph having the lower lattice free energy is the more stable form and it has the lower solubility while that having higher energy is less stable but tends to dissolve faster and has higher solubility [1]. Based on litera-

ture solubility data *Pudipeddi* and *Serajuddin* [12] published a survey that the ratio of metastable/stable polymorph solubility is typically less than 2, though occasionally higher ratios can be observed as reported for sulindac (7x difference between From I and II) [13] and for premafloxacin (23x) [14], etc. Similar trend was found for anhydrate/hydrate solubility ratio (~2) with many exceptions, while the amorphous/crystalline forms solubility differs with factor ~10, generally. Solubility difference of polymorphs will have effect on the bioavailability/bioequivalence (BA/BE) of a drug product if the solubility is the rate limiting factor upon absorption (for BCS II and IV drugs).

Solubility characterized by the equilibrium solubility data is the concentration of the compound in a saturated solution when solid and solute are at equilibrium. This parameter can be measured by several methods however the "gold standard" is still the saturation shake-flask technique (SSF). Recently Avdeef *et al*. published a consensus-based "white paper" commentary which summarized the recommendations for solubility measurement including SSF and other methods to improve the data quality [15]. The paper pointed out to the difficulties in the measurement of polymorphs due to the possible polymorph transitions upon equilibration and the importance of the analysis of both solution and solid phases. Well established methods for solid phase analysis (X-ray powder diffraction XRPD, differential scanning calorimetry DSC, FT-IR and Raman spectroscopy, etc.) should be used for characterization of the solid at the beginning and also at the end of the solubility experiment. Adhering and applying all these recommendations of the "white paper" one can measure only the thermodynamic solubility of that crystalline form which is in dynamic equilibrium with its solution form, at the temperature of the experiment. However, during the dissolution process polymorphs can show different kinetics, resulting in substantially various supersaturation solutions even so if later they convert to another form. The incubation time in the standard protocol of SSF is 24 h (6 h agitation and 18 h sedimentation) [16]. Since solvent induced changes in solid form structure might occur much faster or slower than this timeframe the *in situ* monitoring of the dissolution-time profile may provide more information about the behavior of polymorphs and may enable the optimization of incubation time. Moreover *in situ* monitoring with fiber optic UV probes under certain conditions (low amount of excess solid, long analysis time, etc) enables the determination of dissolution kinetics and also equilibrium solubility from a single experiment.

The goals of the present study were: (1) investigation of equilibrium solubility of different polymorphs of venlafaxine hydrochloride (VENL) as model compound, in wide pH range by SSF method coupled with solid phase analysis; (2) real time monitoring of the dissolution profile using *in situ* UV fiber optic; (3) to compare the equilibrium solubility results obtained by SSF and μDISS methods and (4) to reveal the advantages and limits of the latter technique for solubility measurement.

2. Material and methods

2.1. Materials

The structure of model compound is presented in *Figure 1*. Venlafaxine hydrochloride (313.86 g/mol) was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). From the commercially available polymorph the other polymorphic form was prepared in house based on patent (US6924393B2) and verified by XRPD. Distilled water of Ph. Eur. grade was used. All other reagents were of analytical grade.

A Britton-Robinson (BR) buffer stock solution (a mixture of acetic acid, phosphoric acid and boric acid, each at 0.04 M) was prepared, and the required amount of 0.2 M NaOH or 1M NaOH was added to give the pH specified for the solubility experiments. BR buffer solutions were used in pH range 7.5-11, while at pH 11 and above NaOH was applied.

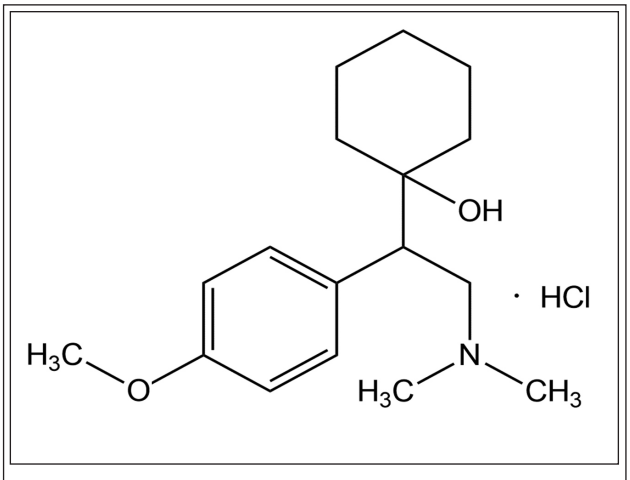

Figure 1 *Structure of venlafaxine hydrochloride*

2.2. Determination of the thermodynamic solubility by saturation shake-flask method (SSF)

2.2.1. Preparation of saturated solution

The equilibrium solubility of VENL in the examined pH region was determined by the SSF method [16, 17]. The sample was added in excess to the aqueous buffer solutions to produce a suspension. The amount of solid added was accurately measured: 10-300 mg/4 mL. At controlled temperature of 25.0 ± 0.1 °C the solution containing solid excess of the sample was vigorously stirred for a period of 6 h (agitation time), what followed a further 18 h of sedimentation period (stirrer turned off).

2.2.2. Concentration determination of the saturated solution

2.2.2.1. Off-line: sampling, dilution and concentration measurement with UV spectrophotometer

The concentration of the saturated solution was measured by UV spectroscopy using JASCO V 550 UV/VIS spectrophotometer. Three aliquots were carefully withdrawn from the liquid, using a fine glass pipette, and were diluted with the solvent if necessary. Three replicate solubility measurements were carried out at each of the tested conditions.

The specific absorbance ($A^{1\%}_{1cm}$, the absorbance of 1 g/100 mL solution over a 1 cm optical pathlength at a given wavelength) of VENL was determined separately at pH 8.0 using 12 points of two parallel dilution series, from the linear regression equation (Lambert-Beer law) where the regression coefficient (r) was higher than 0.9998. The specific absorbance data obtained is 38.4 at λ_{max} 273.5 nm. This value was used for concentration calculation at all pHs since the UV spectrum of VENL is not pH-dependent due to the distance between the protonation site (aliphatic N atom) and the chromophore in the structure.

2.2.2.2. In situ: with fiber optic UV probe

µDISS Profiler™ (Pion Inc. Billerica MA, US, *Figure 2*) has been applied as UV fiber optic instrumentation. The equilibrium concentration was concomitantly determined *in situ* in the equilibrated solubility suspension (without dilution) immersing the UV dip probe. Calibration was performed with the same (2-5-10-20 mm) tip adjusted to the fiber optic UV dip probe which was used in the given solubility experiment. Solubility values obtained from the same experiments analysed with both the *off-line* and the *in situ* UV method were compared (see section *3.3.*).

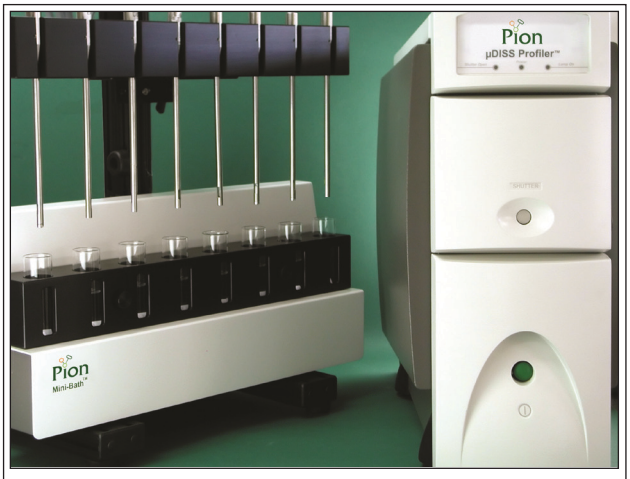

Figure 2 *µDISS Profiler™*

2.3. In situ real time monitoring of dissolution, supersaturation, precipitation and the equilibrium solubility

The drug solution concentration *versus* time (0-24 h) was investigated with the µDISS Profiler™. The instrument measures the real time concentration with fiber optic UV dip probe inserted in 6 temperature controlled 20 mL vessels, stirred with magnetic stirrer. Dissolution of VENL was registered in BR buffer pH ~ 11.5, in three parallel channels, where 10 mg sample was added to 4 mL dissolution media. Using the appropriate 2 mm tip adjusted to the UV probe, the UV spectra were registered in the following timing protocol: per 1 min in 0-2 h, per 10 min in 2-6 h and per 30 min in 6-24 h periods. The stirrer was turned off at 6 h. For the evaluation of the concentration previously determined calibration data and second derivative spectra were used.

2.4. Methods for the analysis of the solid phase

2.4.1. XRPD

Small amount of the solid phase at the end of solubility measurement was isolated and dried to a glass plate. X-ray powder diffraction patterns of these samples were recorded by means of a PANalytical (Amelo, The Netherlands) X'pert ProMDP X-ray diffractometer using Cu-K"α radiation (1.524 Å) and a Ni filter. The applied voltage

was 40 kV, while the current was 30 mA. The samples were analysed between 4° and 42° 2θ.

2.4.2. Raman spectroscopy

Raman spectra were collected using a Horiba Jobin-Yvon LabRAM system coupled with an Olympus 97 BX-40 optical microscope (Olympus Corporation, Tokyo, Japan). The sample was illuminated by a 785-nm diode laser (TEC 510 type, Sacher Lasertechnik, Marburg, Germany) and an objective of ×10 (laser spot size, approximately 4 μm) was used for optical imaging and spectrum acquisition. The laser beam is focused through the objective, and the backscattered radiation is collected with the same objective, a common configuration found in most confocal spectroscopic systems. The collected radiation is directed through an edge filter that removes the Rayleigh photons and then through a confocal hole (500 μm) and the entrance slit (100 μm). Finally, a 950 groove/mm grating monochromator disperses the Raman photons before reaching the CCD detector. Each spectrum of the image was collected in the spectral range of 300 to 1600 cm^{-1}.

2.4.3. Polarized light microscopy

Polarized light microscopic images were taken using Amplival (Carl Zeiss, Jena, Germany) type microscope coupled with Olympus C4040 Z type camera (Olympus, Japan).

2.5. Statistical analyses

Concentrations were expressed as means ± SD, and were compared using "two-sample" Student's *t* test. Differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

In this study we measured the equilibrium solubility of different polymorphic forms of venlafaxine hydrochloride modelling the possible behaviour of a pharmaceutical solid. The pH-dependent equilibrium solubility (S_{pH}) over a wide pH range was investigated by SSF method at 25 °C temperature. The solubility of the free base form was depicted by the intrinsic solubility (S_o) measured at high pH value. To identify the solid form that is present at the solubility equilibrium, the approach was completed with the solid phase analysis at the end of the solubility experiment. We also studied the dissolution kinetics and the extent of supersaturation and the incubation time needed to reach the equilibrium, using real time monitoring by μDISS Profiler™ to reveal the differences between the stable and less stable/metastable forms.

Venlafaxine is a 2nd generation, SNRI (serotonin-norepinephrine reuptake inhibitor) antidepressant drug nowadays widely used in depression, in generalized anxiety disorder, post-traumatic stress disorder, etc. (US Patent, 2006, US7030164B2). VENL is used as racemate of its hydrochloride salt which is known to exist in two polymorphic forms, Form I and Form II. Though Form I is more stable (melting temperature: 210-212 °C, ΔH: 125.8 J/g) than Form II (melting temperature: 208-210 °C, ΔH: 130.3 J/g) the latter is preferred in the formulation because it has larger particle size with better filtration and drying characteristics [18]. VENL is a monovalent base (pK_a: 9.63), its solubility is pH-dependent. Since the compound belongs to BCS I class, the solubility is not an absorption limiting factor from GI tract. Investigation of salt solubility in biorelevant medium was not the goal of this study. However VENL is a good model for studying the pH-dependent solubility of polymorphs and for comparison the SSF and μDISS methods in the measurement of intrinsic solubility.

Table I *Equilibrium solubility of venlafaxine hydrochloride polymorphs in BR buffer measured by SSF method*

VENL Form I			VENL Form II		
pH	**S_{pH} ± SD (mg/mL)**	**n**	**pH**	**S_{pH} ± SD (mg/mL)**	**n**
7.50	59.35 ± 8.23	9	7.54	54.32 ± 4.43	3
7.70	32.08 ± 2.60	9	7.60	45.67 ± 2.34	9
7.83	23.34 ± 0.45	3	7.67	38.35 ± 2.99	9
8.30	8.655 ± 0.267	9	7.78	26.79 ± 0.27	3
8.34	7.194 ± 0.043	3	8.20	8.175 ± 1.061	9
8.37	7.495 ± 0.601	3	8.40	7.689 ± 0.345	9
8.43	6.983 ± 0.103	3	8.52	5.506 ± 0.104	3
8.47	6.364 ± 0.071	3	8.58	4.985 ± 0.053	3
9.30	0.948 ± 0.031	9	8.64	4.587 ± 0.013	3
9.50	0.796 ± 0.094	9	8.70	3.809 ± 0.104	3
9.72	0.574 ± 0.011	3	9.45	0.831 ± 0.027	9
9.76	0.507 ± 0.004	3	9.76	0.567 ± 0.014	4
9.81	0.559 ± 0.062	3	9.96	0.544 ± 0.011	3
9.96	0.570 ± 0.013	3	10.06	0.495 ± 0.052	4
10.34	0.521 ± 0.186	3	10.26	0.523 ± 0.037	4
10.41	0.454 ± 0.011	3	10.41	0.498 ± 0.034	4
11.51	0.275 ± 0.004	4	11.62	0.296 ± 0.025	4
12.11	0.292 ± 0.007	4	12.01	0.285 ± 0.004	4

Concentration is expressed as free base equivalents.

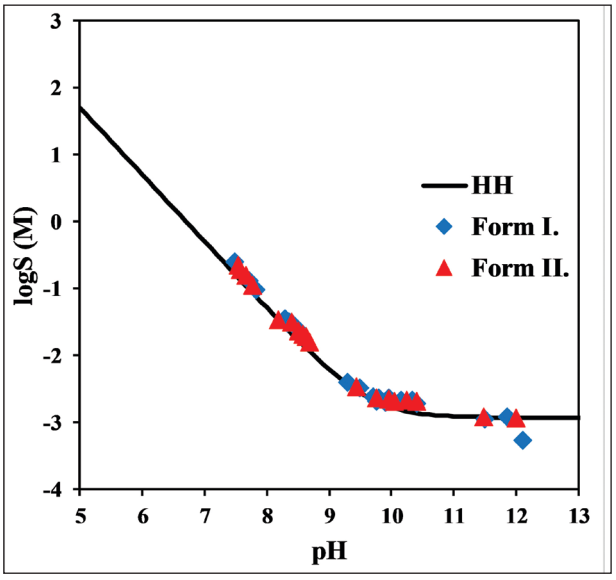

Figure 3 *Solubility-pH profile of VENL Form I and Form II (solid line represents the theoretical HH curve calculated from pKa: 9.63 and logSo: -2.93, points represent the experimental data)*

3.1. Solubility-pH profile of venlafaxine polymorphs

The equilibrium solubility of VENL Form I and II were measured by the standard protocol of SSF method at 18 different points in pH range 7.5-12, at 25 °C. The S_{pH} ± SD results are expressed in mg/mL unit as average value of parallel measurements and are presented in *Table I*. The standard deviation was in the range of 1-13 %, average SD: 6 %. No significant difference was found between the S_{pH} values of polymorphs at the same pH in the whole examined region. This can be interpreted with the transition of polymorphs to a common product as supported by the solid phase analysis (see below *3.2*).

Figure 3 shows the solubility-pH profile of VENL Form I and Form II as plotted logS [M] values *vs* pH. As it can be seen in *Figure 3* the curve is typical for a monoprotic base as described by the theoretical Henderson-Hasselbalch (HH) relationship ($\log S = \log S_0 + \log(10^{pKa-pH} + 1)$ [17, 19].

The intrinsic solubility of VENL base (calculated as the average of values measured at pH ≥ 11.5) is $\log S_o$ [M]: -2.93 ± 0.014 (n=4). This value was used to generate the HH curve of VENL (solid line on *Figure 3*). The experimental data points closely follow the theoretical HH curve. Below pH 10 the VENL free base starts to convert to cationic form and the solubility is increasing. Below pH 7 the solubility was not studied because the salt solubility is very high (> 500 mg/mL) and its saturated solution can be considered non-ideal solution.

3.2. Characterization of the solid phase isolated from solubility experiment

First the starting materials, Form I and Form II were identified by powder X-ray diffraction (see in *Figure 4*). Next the samples isolated and dried from the solubility suspension after incubation at different pHs were studied. Diffractograms shown representatively in increasing pH order in *Figure 4* demonstrate that the samples are not identical with the starting polymorphic forms in neither case. Results indicate that both polymorphs re-

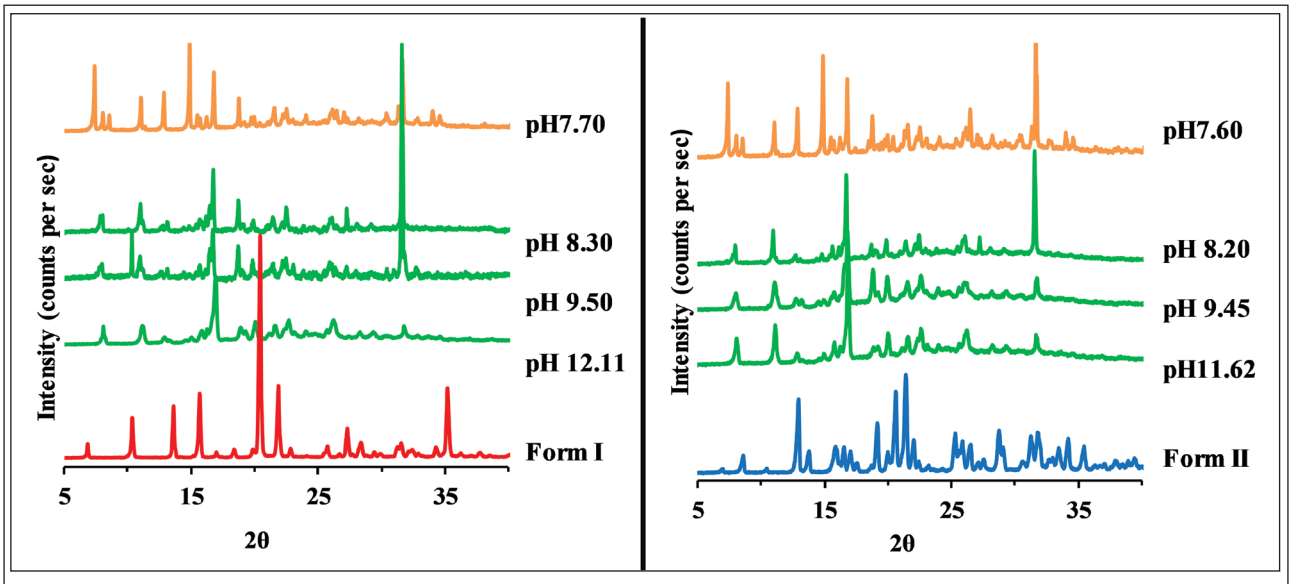

Figure 4 *XRPD patterns of VENL Form I and Form II and the solids isolated from solubility suspensions at different pH values*

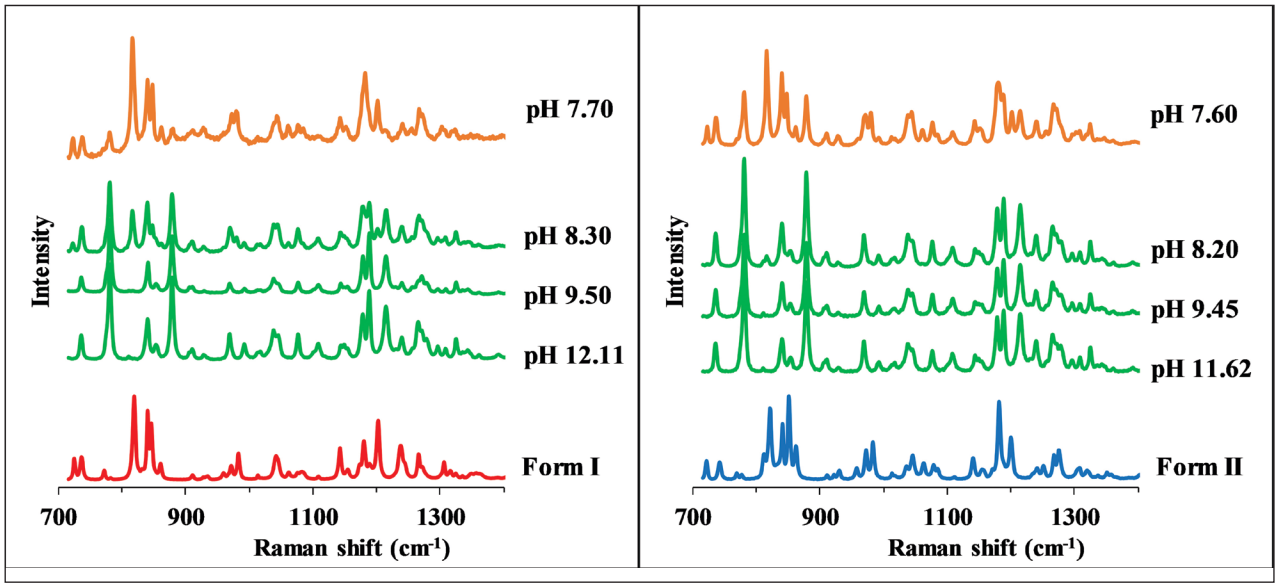

Figure 5 *Raman spectra of solid phase of VENL polymorphs isolated from solubility suspensions*

crystallized to a common form. Product obtained at lower pHs (7.5-9) could not be identified using Cambridge Structural Database. Based on patent US20030114536A1 we identified the product as venlafaxine hydrochloride monohydrate. Patterns registered at higher pH (11-12) values were found identical with one of the free base VENL polymorphs, specified in the database as free base Form I. A peak (if any) appeared with various intensity in the patterns of free base at 9 (2 Theta) is due to the presence of sodium acetate remained in some samples. XRPD finding was supported by Raman spectroscopy as well (*Figure 5*). The polarized light microscopic images in *Figure 6* (A and B panels) indicate that crystal shape of Form I and II is column but they substantially differ in their size. Panel C shows the shape of the free base form isolated at pH 12.

All above results of solid phase analysis confirm and can interpret the equilibrium solubility data measured experimentally (*Table I*) in pH range 7.5-12.

3.3. Comparison of concentration measurement with off-line UV method and in situ UV probe

µDISS Profiler™ measures the concentration of the solute *in situ* in the saturated solution in the presence of solid. In order to prove that it has no impact on the results (e.g. distorting the absorption measurement), in a part of SSF experiment at the end of incubation time the concentration of the saturated solution was measured by two ways: (1) after the necessary dilution with traditional UV spectrophotometer (UV-JASCO), and (2) *in situ* in the solubility vessel (without dilution) by UV probe (UV-µDISS). Solubility values shown in *Table II* are in good agreement. Difference between the results

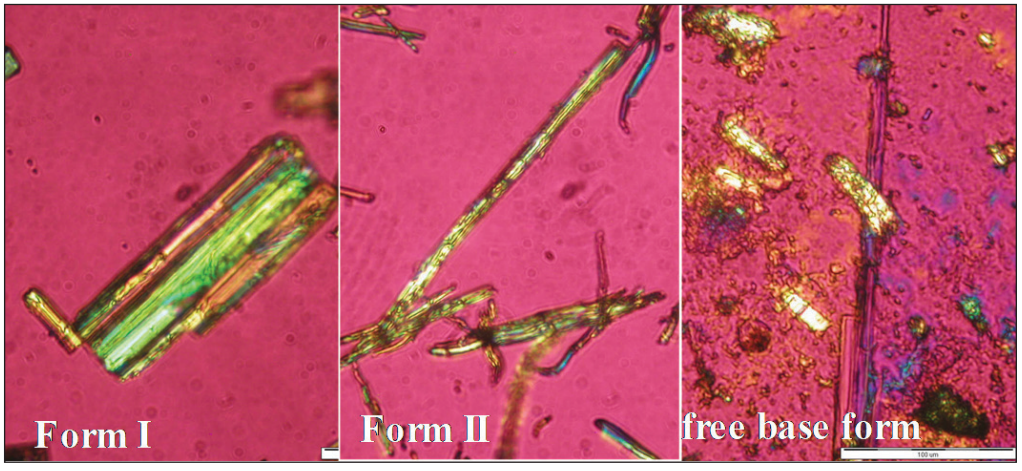

Figure 6 *Polar microscopic images of VENL samples (A: Form I, B: Form II, C: free base isolated at pH 12)*

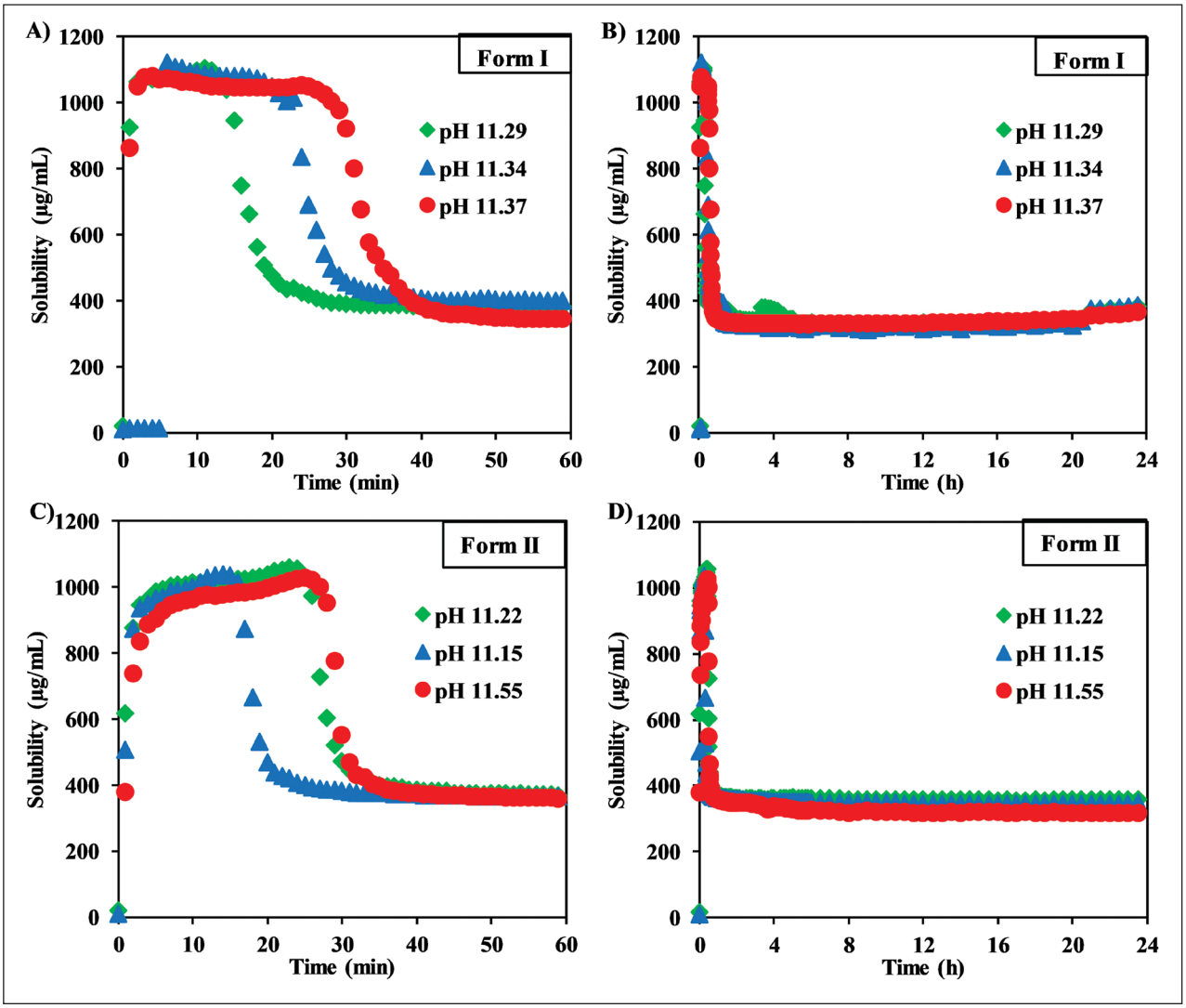

Figure 7 *Solubility-time profile of VENL Form I (upper panels) and Form II (lower panels) at pH ~ 11.5 in BR buffer monitored by µDISS approach*

Table II *Equilibrium solubility of venlafaxine hydrochloride polymorphs measured by SSF method using different concentration detection*

Sample VENL	pH	S_{pH} ± SD (mg/mL)	
		UV-JASCO	UV-µDISS
Form I	7.50	59.35 ± 8.23	56.98 ± 6.89
	7.70	32.08 ± 2.60	31.34 ± 2.40
	7.83	23.34 ± 0.45	22.44 ± 0.31
	9.30	0.948 ± 0.031	1.121 ± 0.038
	9.50	0.796 ± 0.094	0.764 ± 0.080
Form II	7.54	54.32 ± 4.43	48.51 ± 6.32
	7.60	45.67 ± 2.34	45.54 ± 1.25
	7.67	38.35 ± 2.99	36.49 ± 0.45
	7.78	26.79 ± 0.27	26.96 ± 0.40
	9.45	0.831 ± 0.027	0.853 ± 0.030

Concentration is expressed as free base equivalents.

varies in 0.2-15%, average is about 5%. It can be observed that in 7/10 cases µDISS measures the lower value.

3.4. Real time monitoring of dissolution by µDISS Profiler™

The real time dissolution profile was monitored by µDISS Profiler™. It was performed in BR buffer pH ~11-11.5 (exact pH was measured at the end of the experiments) in 0-24 h period, where in the first 6 h stirrer was turned on. The UV spectra were registered according to a protocol (see in section 2.3.) in three parallel vessels and the concentration was calculated using calibration. *Figure 7* shows that dissolution process of VENL can be followed well and the equilibrium solubility can

also be measured by this approach. In case of Form I (*Figure 7* upper panels) the supersaturation is very similar in the three parallel experiments and solute reaches 3.5 times concentration relative to the equilibrium solubility. Precipitation starts at different time in the parallels (12-25-30 min, respectively) and the samples are reaching the equilibrium in rather short time (20-40 min). The concentration is not changing further when at 6 h stirrer is turned off up to 20 h. A small (~13%) increase in concentration can be observed in 20-24 h period for one of the channels. It may be due to the adsorption of solid crystals to the UV probe without stirring of the suspension. The dissolution-time profile of Form II as starting material (*Figure 7* lower panels) is very similar. It reaches a bit slower the same supersaturation as Form I but the precipitation and time needed to reach the equilibrium is the same. We can conclude from this profiling that VENL polymorphs are converting to the common free base form immediately after dissolution. Intrinsic solubility measured by μDISS Profiler™ at 24 h is 0.290 ± 0.004 mg/mL ($logS_o$ [M]: -2.98) (n=4), it perfectly agrees with S_o obtained by SSF method.

4. Conclusions

In this study we have demonstrated the importance of solid phase analysis in the solubility investigation of drug polymorphs giving reliable information about which form is present in solubility equilibrium. We presented an example for the possible behavior of pharmaceutical compounds.

Venlafaxine hydrochloride Form I and Form II transformed to a common product in aqueous buffer solution in pH 7.5-12 region and thus the measured solubility values at a given pH were identical. Solid isolated from pH 10-12 was identified as the free base, while between pH 7-8 the isolated common product was identified as VENL hydrochloride monohydrate based on XRPD results. Since VENL dissolves freely in acidic medium not the solubility but the better manufacturability can explain why the less stable Form II is preferably used in tablets.

We have also presented the good agreement of equilibrium solubility measured by SSF coupled with *off-line* analytics and *in situ* fiber optic concentration monitoring. μDISS Profiler™ has the advantage to provide much more information about dissolution process, with this approach the dissolution kinetic, the supersaturation and the time needed to reach the equilibrium can be easily monitored. It is simpler and occasionally can be faster than traditional methods however there are limits of its application. Neither too high nor too low solubility can be measured with this instrument, the optimal solubility window is about 1-500 μg/mL. Since the new APIs in drug research are mainly poorly soluble compounds, for their solubility study μDISS Profiler seems to be advantageous.

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