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SCIENZE CHIMICHE

CICLO XXXI

Crystallization studies and identification of crystalline forms suitable for inhalation in drug discovery

Coordinatore:

Chiar.mo Prof. Roberto Corradini

Tutore:

Chiar.mo Prof. Alessia Bacchi

Correlatori:

Dott. Francesco Amadei

Chiar.mo Prof. Luciano Marchiò

Dottorando:

Valentina Diana Di Lallo

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Abstract

This thesis work is the result of an industrial Ph.D in collaboration with Chiesi Farmaceutici S.p.A., a pharmaceutical company with a strong expertise in the research and development of drugs for inhalation route.

The work carried out during the three years was focused on the study of solid state properties and polymorphism of different molecules of industrial interest. In particular, those studies emphasized the efforts put in the identification of suitable crystalline forms of New Chemical Entities (NCEs) in a very early phase of drug development and in the studies of their solid state properties.

The aim of the manuscript is to review the state of the art of academic and industrial knowledge about the design and characterization of solid state forms of NCEs, in order to contribute some guidelines and present selected information which can be useful to industrial research laboratories which do not have usually a deep insight into crystallography.

During this thesis work, these concepts were applied during the laboratory practice, mixing approaches and techniques well known and established in the pharmaceutical industry with some others peculiar to the academic world.

Only a general description of the studies performed on different molecules of industrial interest will be here reported. All the experimental results obtained from the different studies have to be considered confidential and no one of these is reported in this thesis.

Riassunto

Questo lavoro di tesi è il risultato di un dottorato di ricerca industriale svolto in collaborazione con Chiesi Farmaceutici S.p.A., un'azienda farmaceutica con una forte competenza nella ricerca e sviluppo di farmaci per la somministrazione inalatoria.

Il lavoro svolto durante i tre anni si è concentrato sullo studio delle proprietà di stato solido e polimorfismo di diverse molecole di interesse industriale. In particolare, lo scopo di tali studi era volto a sottolineare il particolare impegno che si richiede per l'individuazione di adeguate forme cristalline di nuove entità chimiche (NCEs) in una fase molto precoce dello sviluppo di farmaci e negli studi di loro proprietà allo stato solido.

L'obiettivo del manoscritto è di esaminare lo stato dell'arte della conoscenza accademica e industriale sulla progettazione e caratterizzazione di forme solide di NCEs, al fine di contribuire con alcune linee guida ed informazioni che possano essere utili per laboratori di ricerca industriale, che non hanno solitamente una profonda conoscenza di cristallografia.

Durante questo lavoro di tesi, si è cercato di applicare le conoscenze ottenute nel quotidiano lavoro di laboratorio miscelando approcci e tecniche ben note nel settore farmaceutico con altre peculiari del mondo accademico.

Per motivi di confidenzialità e segretezza, solo una descrizione generale degli studi eseguiti sulle diverse molecole di interesse industriale verrà riportata. Tutti i risultati ottenuti dai diversi studi sono infatti da considerarsi riservati e perciò nessuno di essi è riportato in questa tesi.

Contents

Introduction

1. Introduction

Inhalation treatment of lung diseases began in the early 1950s when the first inhaled drug for asthma therapy emerged [1,2]. Since ever, significant inhalation products have been developed for the treatment of asthma, and also for other pulmonary diseases, such as chronic obstruction pulmonary diseases (COPD), cystic fibrosis, pneumonia. The rationale for such treatments includes more localized delivery, direct to the desired target organ, with minimum systemic exposure [3,4]. More recently, attention has been placed on systemic delivery of drugs administered by inhalation due to its evident advantages, such as:

- enormous surface area of the lungs (ca 100 m^2 in adults)
- good epithelial permeability
- extensive vascularization
- faster onset of action compared to the oral route
- avoidance of first pass metabolism

For these reasons, a variety of inhalation products are now under development for treatment of systemic diseases also. The current inhalation products on the market or undergoing clinical studies, their drug classification and therapeutic usage are summarized in Figure 1: Current inhaled pharmaceuticals for treatment of lung diseases on the market or undergoing clinical studies. Reprinted from [3].

(for local treatment of lung disease, the majority) and Figure 2 (for systemic application) [4,5].

For effective inhalational drug delivery, physical properties of the particles are critical. For efficient deposition in the lungs, the particles should have an ideal particle size and morphology that provide optimal aerodynamic performance. Moreover, these particles should avoid uptake by alveolar macrophages (unless these are not the intended target). Finally, the ideal dry particles should maintain physical and chemical stability during storage. Therefore, an early identification of a robust crystalline form is highly

recommended, and many efforts are applied since the last phases of drug discovery to select and characterize proper solid forms.

Therapeutic usage	Drug classifications	Drugs	Inhalation device	Current development status
COPD and asthma	Short-acting beta-2 agonist (SABAs)	Salbutamol (albuterol)	Nebulizer, pMDI, DPI	Marketed
		Fenoterol	pMDI	Marketed outside of US
		Pirbuterol	Nebulizer, pMDI	Discontinued by Dec.2013
		Terbutaline	DPI	Marketed
		Levalbuterol	pMDI	Marketed
	Long-acting beta-2 agonist (LABAs)	Salmeterol	pMDI, DPI	Marketed
		Formeterol	pMDI, DPI	Marketed
		Arformoterol	Nebulizer	Marketed
		Indacaterol	DPI	Marketed
	Anticholinergic agents	Ipratropium bromide	Nebulizer, pMDI	Marketed
		Tiotropium bromide	pMDI, DPI	Marketed
		Aclidinium bromide	DPI	Marketed
		Oxitropium bromide	pMDI	Outside of US
		Glycopyrronium bromide	DPI	Outside of US
	Inhaled corticosteroids (ICS)	Beclomethasone dipropionate	Nebulizer, pMDI, DPI	Marketed
		Budesonide	Nebulizer, DPI	Marketed
		Fluticasone propionate	pMDI, DPI	Marketed
		Mometasone furoate	pMDI, DPI	Marketed
		Ciclesonide	pMDI	Marketed
	Combination therapy	Fenoterol/Ipratropium	pMDI	Marketed
		Salbuterol/Ipratropium	pMDI	Marketed
		Formeterol/Budesonide	pMDI, DPI	Marketed
		Formeterol/Mometasone	DPI	Marketed
		Salmeterol/Fluticasone	pMDI, DPI	Marketed
		Glycopyrronium/formoterol	pMDI	Phase II
	Sugar alcohol	Mannitol	DPI	Marketed
	Antisense	AIR-645	Nebulizer	Phase II
	Oligonucleotides	PXSTPI-1100	Nebulizer	Preclinical
		ATL-1102	Nebulizer	Preclinical
	CpG oligonucleotides	QAX-935 (IMO-2134)	Nebulizer	Phaes I
	siRNA	Excellair	Nebulizer	Phase II
Cystic fibrosis	Antibiotics	Tobramycin	Nebulizer, DPI	Marketed
		Aztreonam	Nebulizer	Marketed
		Colistimethate sodium	Nebulizer	Pilot trials
		Liposomal ciprofloxacin	Nebulizer	Phase II
		Liposomal amikacin	Nebulizer	Phase III
		Levofloxacin	Nebulizer	Phase III
		PUR118	DPI	Phase I
	Mucous mobilizers	Dornase alfa	Nebulizer	Outside of US
		Lancovutide	Nebulizer	Phase II
	Restore Airway Surface Liquid	Hypertonic saline	Nebulizer	Marketed
		Mannitol	DPI	Phase III
	Antiproteases	Alpha$_1$-antitrypsin	Nebulizer	Phase II
	MRSA lung infections	Vancomycin	DPI	Phase II
Respiratory distress syndrome	Pulmonary surfactant	Phospholipids/surfactant proteins	Endo-tracheal tube	Marketed
Respiratory Syncytial Virus	Antiviral	MDT-637	Novel inhaler	Phase II

Figure 1: Current inhaled pharmaceuticals for treatment of lung diseases on the market or undergoing clinical studies. Reprinted from [3].

Therapeutic usage	Drug classifications	Drugs	Inhalation device	Current development status
Analgesia	Opioids	Fentanyl	Novel inhaler	Phase II
		Liposomal fentanyl	Nebulizer	Phase II
Migrane	Triptan	Sumatriptan	Intranasal powder	Phase III
Diabetes	Peptides	Insulin	DPI	Phase III
		Glucagon-like peptide	DPI	Phase I
Nerve gas poisoning	Nerve agent antidote	Atropine	Novel inhaler	Phase I
Parkinson's disease	Psychoactive drug	Levodopa	DPI	Phase II
Schizophrenia	Antipsychotic medication	Loxapine	DPI	Outside of US

Figure 2: Current inhaled pharmaceuticals for systemic application on the market or undergoing clinical studies. Reprinted from [3].

During this thesis work the most used crystallization techniques were applied in order to investigate the solid state properties of the New Chemical Entities (NCEs) objects of the research. Due to confidentiality reasons no experimental data and results can be shared, so this thesis work aims to generally discuss about the emerging applications of crystallography in the pharmaceutical field, specifically with regard to the selection and characterization of crystalline forms suitable for inhalation as Dry Powders, taking advantage of the most recent innovative techniques available for implementation in a company.

Starting from a brief insight in the available platforms for inhalation drug delivery, in pulmonary physiology and in the general requirements of inhalable dry particles, then an excursus of the crystallization and the principal crystallization techniques in this field is provided, from the conventional ones mainly used in industrial applications to some which are more studied in the academic field.

1.1. Inhalable Drug-Delivery Systems

Three platforms of inhalable delivery systems are widely used in pulmonary drug delivery, the first two for liquid formulations, the last one for solid formulation (Figure 3):

- Pressurized Metered Dose Inhalers (pMDI)
- Nebulizers for liquid formulations
- Dry Powder Inhaler (DPI).

The present work is focused on the evaluation of the crystallographic properties of drugs for pulmonary administration via DPI, but a brief description of all the platforms is reported below.

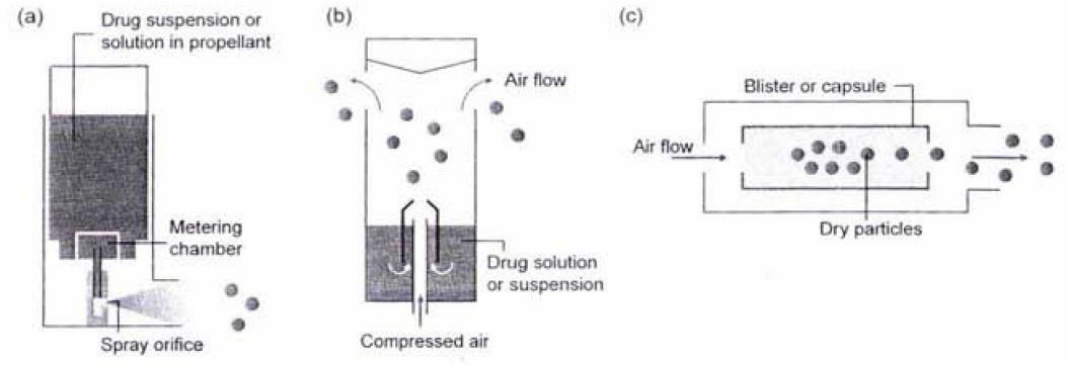

Figure 3: An illustration of devices for pulmonary drug delivery: (a) pMDI, (b) nebulizer, and (c) DPI. Reprinted from [6].

The metered dose inhaler is one of the most widely used methods of aerosol drug delivery because of its reliability and low cost. However, its use is often limited to the treatment of upper airway, conditions due to low drug deposition in the lungs and formulation challenges with some peptide based drugs. Drugs delivered by pMDIs are usually prepared as suspensions or solutions in a propellant, which often contain co-solvents or surfactants to assist in the dispersion of drugs. The drug aerosol is created by releasing a small volume of pressurized drug dispersion from a metering chamber through a spray orifice. As the released drug dispersion begins to equilibrate with the atmospheric pressure, it is propelled out of the container, forming a spray of droplets [6,7].

The nebulizer platform atomizes an aqueous based drug solution by air jet or ultrasonic mechanisms. It is typically used for delivering doses over multiple breaths, and to infants, elderly and critically ill patients. Compressed-air nebulizers are usually less efficient than pMDIs in production and delivery of the aerosol, and their portability is limited. However, advances in nebulizer technology have overcome some of the historical limitations. Nebulizers remain attractive due to the independence of aerosol generation from patient inhalation coordination, and relative easy formulation handling [6,7].

The dry powder platform is constituted by a collection of dry particles contained in an inhaler device. The powder is usually composed of a micronised Active Pharmaceutical Ingredient (API) (1-5 µm) and carrier excipients, which are added to improve the dispersibility of the drug. Unfortunately, the cohesiveness of the fine particles due to strong inter-particle forces makes them very difficult to process and dispense. Also, they tend to form agglomerates, which are difficult to break up into the desired primary particles for optimal deposition in the lung [7,8,9].

In general, a patient's DPI dose is dependent on four interrelated factors:

- the properties of the drug formulation, particularly powder flow, particle size, shape and surface properties and drug carrier interaction;
- the performance of the inhaler device, including aerosol generation and delivery;
- correct inhalation technique for deposition in the lungs;
- the inspiratory flow rate.

Therefore, a balance among the design of an inhaler device, drug formulation and the inspiratory flow rate of the patient is required [10].

Dry powder inhaler devices can be also classified by dose type into single-unit dose, multi-dose reservoirs, and multi-unit dose, as illustrated schematically in Figure 4 [7].

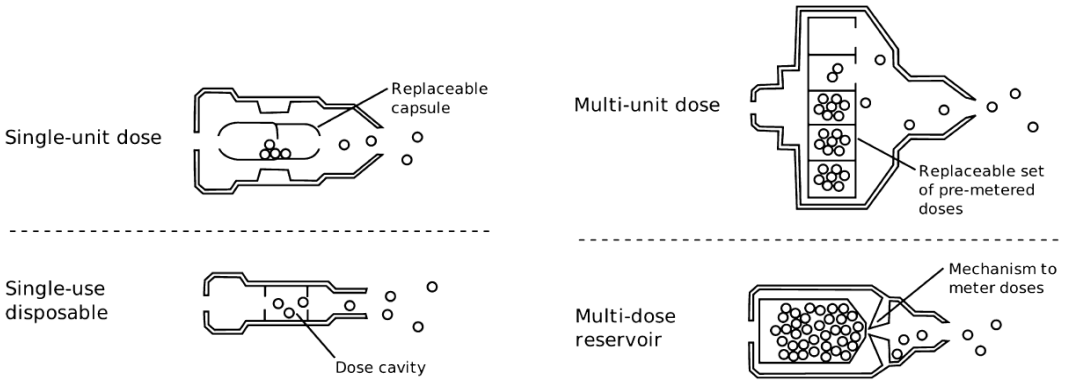

Figure 4: Illustration of four dose design options available for dry powder inhalers. Reprinted from [7].

Finally, Figure 5 shows the different types of formulations strategies employed to formulate drugs in Dry Powder Inhalers [7].

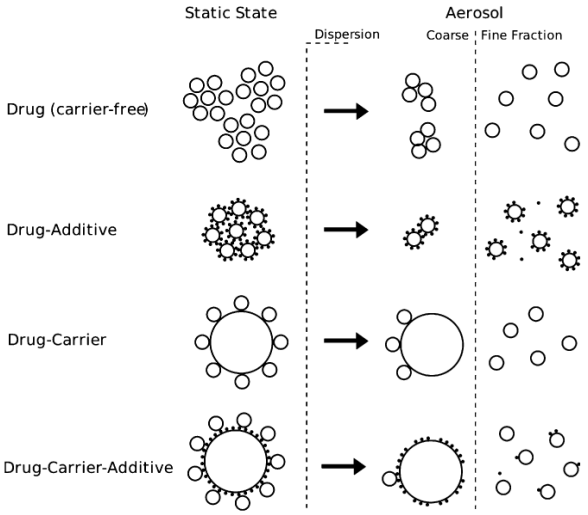

Figure 5: Illustration of the different types of formulation strategies for powders intended for pulmonary drug delivery. Reprinted from [7].

1.2. Physiology of the Lung

Within the lungs, the trachea, bronchi and bronchioles are analogous to the trunk and branches of a tree, whereas the sac-like alveoli can be compared to the leaves. Like in a tree, the airways bifurcate in different branches, roughly 16-17 times before the alveoli are reached, as showed by the classic model described by Weibel reported in

Figure [2,3,11,12].

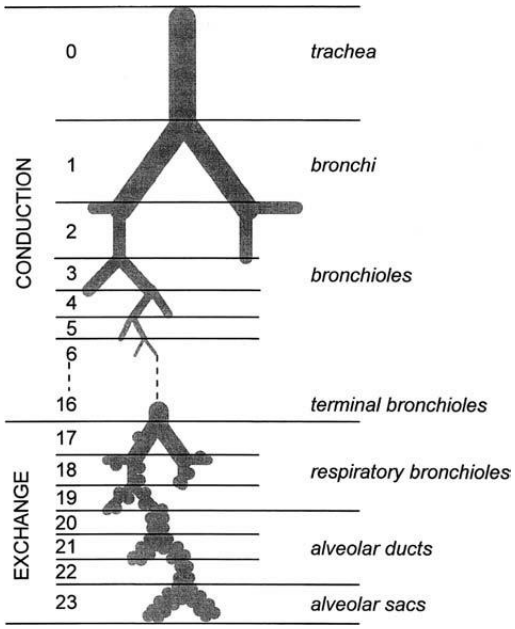

Figure 6: Model of airway according to Weibel. Reprinted from [12]

The airways can be divided in two principal compartments:

- the Tracheo-bronchial region (which is also referred to as the “conductive airways”, or “conductive zone”), which starts at the larynx, and extends via the trachea, bronchi, and bronchioles and ends at the terminal bronchioles;
- the Alveolar region (which is also referred to as the “respiratory airways”, “gas-exchange zone”, “peripheral airways” or “pulmonary region”), which comprises the respiratory bronchioles, alveolar ducts and alveoli.

The surface area of the conductive zone is only a few meters square in the adult human, as compared with the alveolar surface of more than 100 m^2 constituting the peripheral airways. In addition, the two regions substantially differ for the pseudostratified epithelium of cells that constitute the barrier to absorption into the bloodstream, as showed in

Figure [2].

The conductive airways are composed of a gradually thinning columnar epithelium populated by many mucus and ciliated cells that collectively form the mucociliary

escalator. The mucociliary escalator moves the mucous layer and the substances trapped within it toward the oral cavity, where they are either swallowed or coughed up. This mucociliary clearance is a major barrier to pulmonary delivery in this zone.

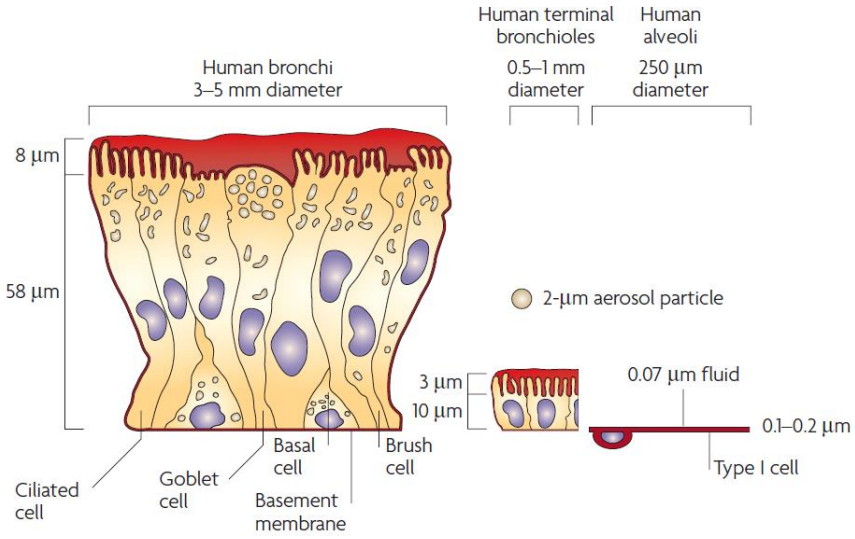

Figure 7: Comparison of the lung epithelium at different sites within the lungs. Reprinted from [2].

The monolayer that constitutes the alveolar epithelium is completely different. Here the tall columnar mucus and cilia cells are replaced primarily (>95% of surface) by the very broad and extremely thin (<0.1 µm in places) type 1 cells. Distributed in the corners of the alveolar sacs are also the progenitor cells for the type 1 cells and the producers of lung surfactant, the type 2 cells. The air-side surface of each of the 500 million alveoli in human lungs is regularly controlled by alveolar macrophages, which engulf and try to digest any insoluble particles that deposit in the alveoli.

1.3. Fundamental Requirements of Inhalable Dry Particles

The global efficiency of any inhalation system derives from the product of the fraction of Emitted Dose (ED), the dose delivered to the lung (i.e., Fine Particle Fraction, FPF) and the lung bioavailability. Both ED and FPF are routinely determined *in-vitro* by means of a multistage cascade impactor (MSCI) and primarily depend upon the particulate properties and inhaler design. FPF is measured as the mass of particles (with reference to the ED) below a certain cut-off diameter; e.g., 4.7 µm, which is below the Andersen Cascade Impactor stage 2, (a cascade impactor operates on the principle of inertial impaction). The bioavailability is influenced not only by the nature of the drug, its *in-vivo* molecular permeability and metabolism, but also by the particle size and shape through their effects on the dissolution rate and phagocytic clearance in the lung [13].

Hence, particle size, shape, density, surface properties, electrical charge and hygroscopicity are important variables in designing an aerosol formulation. In fact, the inhaled particles are deposited at the different levels of the respiratory tract based on their behavior in airflow, which depends on the size, density, and shape of particles and is characterized by the aerodynamic diameter of the particles [10,13,14].

The aerodynamic diameter, which is routinely derived as mass median aerodynamic diameter (MMAD) by sizing techniques that are based on inertial impaction, is defined as the diameter of a sphere with a unit density that has the same terminal settling velocity in still air as the particle in consideration. The aerodynamic diameter d_{ae} is defined through the equation:

$$d_{ae} = d_{geo}\sqrt{\left(\frac{\rho_p}{\rho_0 \chi}\right)}$$

where:

d_{geo} = geometric diameter

ρ_p = particle density

ρ_0 = unit density

χ = dynamic shape factor

The particle density ρ_p of this equation should not be confused with the true density of the dried material. The particle density is the mass of the particle divided by the volume of a sphere of diameter d_{geo} and can be significantly lower than the true density, because it includes internal and external voids [15].

Pharmaceutical powders are rarely spherical, and shape factors are dimensionless measures of the deviation from sphericity. The dynamic shape factor χ is the ratio of the actual resistance force experienced by a non-spherical falling particle to the resistance force experienced by a sphere having the same volume. Consequently, the aerodynamic diameter can be decreased by decreasing the particle size, decreasing particle density, or increasing the dynamic shape factor [10]. The aerodynamical diameter is only a weak function of particle density, which only becomes important if it is significantly lower than unit-density. This explains the current trend to employ particles of very low density on the order of 0.1 g/cm^3 in pulmonary delivery, where a small aerodynamic diameter is desirable [15].

The three principal mechanisms of particle deposition which operate within the respiratory tract are strictly depending upon the aerodynamic diameter. These mechanisms, showed in Figure 8 [10,11], are:

- Impaction
- Sedimentation
- Brownian diffusion

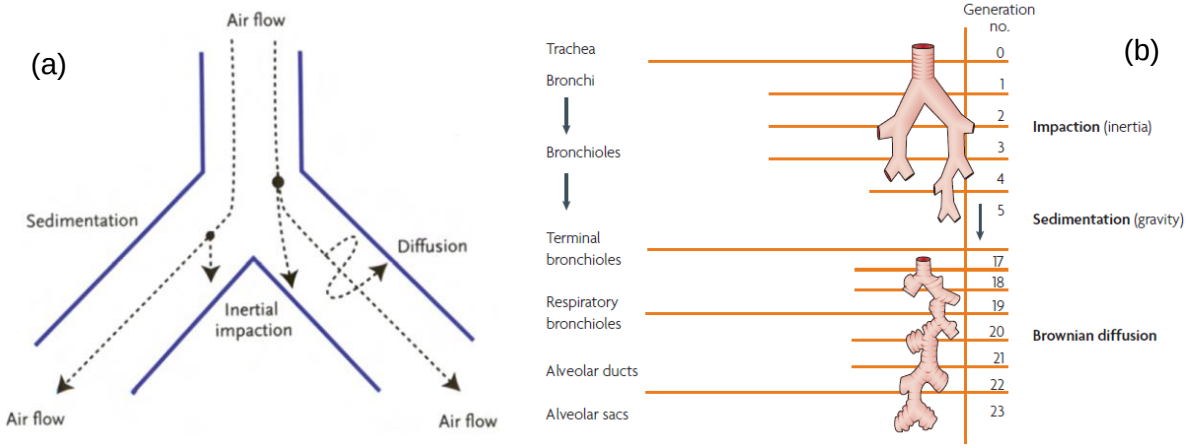

Figure 8: (a) Factors that determine the deposition of inhaled particles. Reprinted from [2]. (b) Description of particle deposition mechanisms at an airway branching site. Reprinted from [11].

Impaction is the inertial deposition of a particle onto an airwaysurface, and is the dominant mechanism for particles with an aerodynamic diameter >5 μm in the upper tracheobronchial regions. These particles may be unable to follow the changing direction of the inspired air as it passes the bifurcations and as a result will collide with the airway walls as they continue on their original course. Impaction therefore usually occurs near the bifurcations. The probability of impaction increases with increasing air velocity, rate of breathing, particle size (>5 μm) and density. Particles larger than 10 μm will impact in the upper airways and are rapidly removed by coughing, swallowing and mucociliary processes.

Particle deposition by sedimentation results from settling under gravity. It becomes increasingly important for particles in the size range 0.5–5 μm. These particles escape impaction and reach airways where the airstream velocity is relatively low, e.g. the bronchioles and alveolar region. To reach the alveolus tissue specifically and therefore obtain systemic absorption, the particles need to be in the range of 1-3 μm. Deposition of these particles increases with longer residence time but decreases as the breathing rate increases.

Brownian diffusion plays a significant role for submicron particles (<1 μm) only. Particles below this size are displaced by a random bombardment of gas molecules, which results in particle collision with the airway walls. The probability of particle deposition by diffusion increases as the particle size decreases. Brownian diffusion is also more prevalent in regions where airflow is very low or absent, e.g. in the alveoli. However, particles of this size are mostly exhaled by the expiratory airflow.

Therefore, to reach the lower respiratory tract and optimize pulmonary drug deposition, aerosols must have aerodynamic diameters between 1 and 5 μm.

Furthermore, the chemical-physical stability and solid state structure of particles are two other key points that need to be carefully considered together with aerodynamic performance and dissolution behavior. Generally, pure solid drug particles used in DPI formulations (and pMDI as well) are required to be crystalline, because they are typically non-spherical, have low-energy surfaces and are stable thermodynamically, and in the most stable form to avoid any potential changes associated with solid state transitions[13]. However, amorphous form has recently gained consideration for the merits of achieving rapid dissolution and absorption, stabilizing biological molecules and/or formulating drugs and bio-therapeutics into sustained-release biodegradable polymeric microspheres or microcapsules.

Due to what above discussed, it is evident that many efforts have been put by pharmaceutical companies and academic world to obtain suitable dry powders with satisfactory pulmonary delivery efficiency. The focus of this thesis work is on the efforts put in the identification of the suitable crystalline forms in a very early phase of drug development and in the studies of their solid state properties. The aim of the manuscript is to review the state of the art of academic and industrial knowledge about the design and characterization of solid state forms of NCE, in order to contribute some guidelines and present selected information which can be useful to industrial research laboratories which do not have usually a deep insight into crystallography.

Crystallization and crystal growth

2. Crystallization and crystal growth

“Structure and function are intimately related” [16].

Crystallography is defined as the study of crystals and their structure. It allows to build models of a crystalline solid where each individual crystal is composed of a single arrangement of atoms or molecules that repeats throughout three-dimensional space.

X-ray crystallography is the most unambiguous and comprehensive way of determining the arrangement of molecules in a crystal structure and it is a method for determining absolute configuration of a molecule. An accurate knowledge of crystal structure is a powerful tool for drug design and functional studies.

In order to know about the positions of the atoms in a crystal, a crystallographer needs to know three things: the unit cell parameters, the space group, and the coordinates of the atoms in the asymmetric unit. With that information, the crystallographer could create a representation of the crystal useful to identify an unknown compound or a detailed geometry to help understanding observed chemical or physical properties.

In the pharmaceutical field the evaluation of the proper crystalline form and the related solid state properties has begun to play a more and more crucial role. In fact, the knowledge of the solid state characteristics of a compound offers more options during the selection of the most suitable crystalline form and also avoids unwanted ‘‘surprises’’ later in development [17].

2.1. What is a Crystal

2.1.1. Definitions

A crystal is comprised of atoms or molecules that repeat throughout three-dimensional space. An ideal crystal is constructed by the infinite repetition in space of identical structural units.

The smallest repeating pattern is called the unit cell; by repeating the pattern of the unit cell over and over in all directions the entire crystal lattice can be constructed. The length of its three edges (a, b, c) and the angles between them (α, β, γ) determine the unit cell size. There are seven basic unit cell geometries, shown in Figure 9, called crystal systems:

- Cubic: all three axes are equal in length and perpendicular to one another
  - $a = b = c$; $\alpha, \beta, \gamma = 90°$
- Tetragonal: two of the three axes are equal in length and all three axes are perpendicular to one another
  - $a = b \neq c$; $\alpha, \beta, \gamma = 90°$
- Orthorhombic: all three axes are unequal in length and all are perpendicular to one another
  - $a \neq b \neq c$; $\alpha, \beta, \gamma = 90°$
- Hexagonal: three of four axes are equal in length and are separated by equal angles and lie in the same plane. The fourth axis is perpendicular to the plane of the other three axes
  - $a = b \neq c$; $\alpha, \beta = 90°$; $\gamma = 120°$
- Rhombohedral (or trigonal): all three axes are of equal length and none of the other is perpendicular to another, but the crystal faces all have the same size and shape
  - $a = b = c$; $\alpha = \beta = \gamma \neq 90°$
- Monoclinic: all three axes are unequal and two axes are perpendicular to each other
  - $a \neq b \neq c$; $\alpha, \gamma = 90°$; $\beta \neq 90°$
- Triclinic: all three axes are unequal in length and none is perpendicular to another
  - $a \neq b \neq c$; $\alpha \neq \beta \neq \gamma \neq 90°$

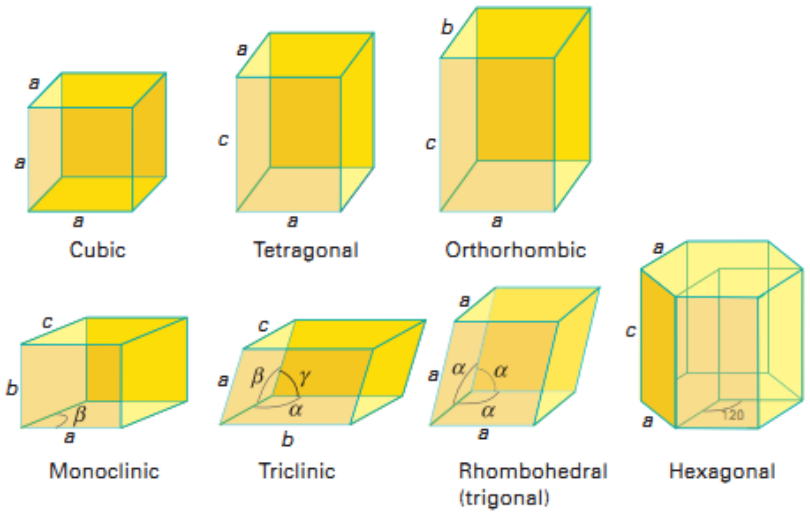

Figure 9: The seven crystal systems [18]

The symmetry is the set of mathematical rules that describe the shape of an object. In a crystal, the symmetry of the repeating pattern is described by the space group of the crystal. A symmetry operation can be defined as an operation which, when applied, results in a structure indistinguishable from the original one. The symmetry of the arrangement of all the individual molecules within the unit cell is described by one of the 230 unique space groups.[19]

There are two types of symmetry that can exist in a crystal.

The point symmetry includes operations such as:

- inversion centres (-1),
- n-fold axes (2, 3, 4, 6),
- improper rotation axis (-3, -4, -6)
- mirror planes (m).

Space symmetry includes:

- unit cell lattice centering
    - P, primitive;
    - C, side-centered;
    - I, body-centered;
    - F, face-centered,

- screw axes (2_1, 3_1, 3_2, 4_1, 4_2, 4_3, 6_1, 6_2, 6_3, 6_4, 6_5),
- glide planes (a, b, c, d or n).

These symmetry operations could be combined and, based on the symmetry in the crystal, a space group is assigned to each crystal structure.

The knowledge of the crystal system and of the space group is of fundamental importance to characterize the solid phase of a NCE. In fact, a compound will in general occur in the most thermodynamically stable arrangement, defined as crystal packing, which minimizes the free energy of the system. However, it often happens that several energetically accessible crystal packing might be observed for a same compound, a phenomenon called polymorphism, which will be described in Chapter 3. Different polymorphs are primarily labelled and distinguished by the different dimensions and symmetry of their unit cells.

The understanding of the factors governing the assembly of molecules, or ions, in a defined crystal arrangement are still an open subject in the scientific arena, and it is nowadays tackled by the discipline defined as crystal engineering.

2.1.2. Crystal engineering

Crystal engineering is the understanding of intermolecular interactions in the context of crystal packing and the utilization of such understanding in design of new solids with desired physical and chemical properties [20]. The intermolecular interactions, such as hydrogen bonds, halogen bonds, coordination bonds, and other less directed interactions, define substructural patterns, referred to in the literature as supramolecular synthons and secondary building units [21].

The crystal is a supramolecular entity *par excellence* [22], and knowledge and control of intermolecular interactions is crucial to understand and design the packing of a solid material. In an ideal situation, a crystal structure is held by sets of robust intermolecular interactions in roughly orthogonal directions, and the crystal engineer should be able to manipulate each set independently. One of the main reasons for understanding the factors which govern crystallization, is that the properties of a solid material depend on the arrangement of the structural units, molecules or ions, in the crystal. Properties like

solubility, dissolution rate, morphology, stability, hygroscopicity, are directly related to the entity and geometric arrangement of the intermolecular interactions in the crystal.

Within the notion of a crystal as a supramolecular entity lie certain key ideas central to the activity of crystal engineering. These are the nature of the crystallization process at a molecular level, crystal packing, molecular interaction and directed molecular recognition. Crystal engineering now encompasses many aspects of solid-state intermolecular interactions, structure prediction, control and rationalization, as well as the synthesis of novel molecular building blocks and crystalline materials. Moreover, crystal engineering has considerable overlap with supramolecular chemistry, X-ray crystallography, materials science, and solid-state chemistry.

Among the properties that could be changed thanks to crystal engineering, the improvement of solubility and dissolution rate is well studied, and of paramount importance for pharmaceutical research, and crystal engineering gives a number of routes which can be adopted through an in-depth knowledge of crystallization processes and the molecular properties of active pharmaceutical ingredients (APIs) [23].

However, the complete and reliable prediction of the result of a crystallization process is nowadays far from being accessible, and therefore a deep knowledge of the tools of crystal engineering is important in order to tackle the problem of understanding the relationships between structure and properties in the most efficient way.

If the crystal is a supramolecular entity, one could argue that the tools of computational chemistry should allow to predict the most stable arrangement of intermolecular interactions of a given set of molecules, thus providing the theoretical thermodynamically stable structure of any given compound, in the same ways as nowadays computational tools allow to correctly predict the geometry of an isolated molecule. However, crystal structure prediction (CSP) which is the computational prediction, from the molecular structure, of the space group and the positional parameters of the atoms in the crystal structure, is far from being a routine tool to obtain the true most stable crystal form of any compound. It is in fact recognized to be a major scientific problem of great difficulty. In a typical CSP experiment, a number of crystal structures are obtained computationally by using a selected force field, and the experimental structure is hidden generally amongst the 100 or so lowest-energy structures [21].

The generation and selection of the correct form among many energetically similar alternatives is a problem not yet solved. This is particularly true for the inhalable drugs due to some of their peculiar characteristics, such as higher polar surface area, higher molecular weight, higher total number of hydrogen-bond acceptors and donors, higher rotatable bond, and subsequently an higher flexibility, compared to their orally administered counterparts. Larger and more complex molecules present major challenges not only in the synthesis but also in crystallizing or in predicting the crystal structures of this molecules [24].

The crystal structure prediction has not been a subject of this thesis work, mostly due to the mentioned issues related to molecules developed for inhalation administration.

2.2. Crystallization screenings

In the pharmaceutical field, choosing and developing the optimal solid form is important not only to obtain a product with the desired physical-chemical properties but also to select a polymorph that can be protected by patents [25]. Depending on the internal packing of their molecules, materials in the solid state can be found in either:

- crystalline: molecules packed in a defined order, which may occur as polymorphic crystals (molecules have different repeating packing arrangements), co crystals (API molecules are stoichiometrically associated to molecular partners, called co-formers, which alter the packing arrangement), solvates (molecules of solvent are stoichiometrically included in the crystal packing), or salts, or any variation and combinations of these [26]
- amorphous: molecules have no long-range three dimensional (3-D) order [25]

Each of these changes in internal packing of a solid will give phase transitions such as polymorph interconversion, desolvation of solvate, formation of hydrate and conversion between crystalline and amorphous forms during various pharmaceutical processes. Those transitions may lead to changes in bulk properties such as physicochemical and mechanical, or alter the dissolution rate and transport characteristics of the drug.

Hence, it is desirable to choose the most suitable and stable form of the drug in the initial stages of drug development [27].

Screening active pharmaceutical ingredients (APIs) to investigate new solid forms is a common practice [28]. Techniques used for screening have evolved over the years to expand the classical screens and/or to deal with complex molecules.

Depending on information needed, different screenings can be performed based on the stage of drug development. Focusing on an early phase of drug development, only some preliminary studies can be performed, also considering the type and amount of material available. As reported in the upper part of Figure 10, during an early phase of drug development a small polymorph, salt or co crystals screening, using additional materials such as counter ions and co-formers, is performed.

Generally, in the latest phases those studies are repeated, increasing the number of experiments. Besides other alternative approaches may be considered, such as the evaluation of alternative formulation strategies, but this is not the focus of this thesis work.

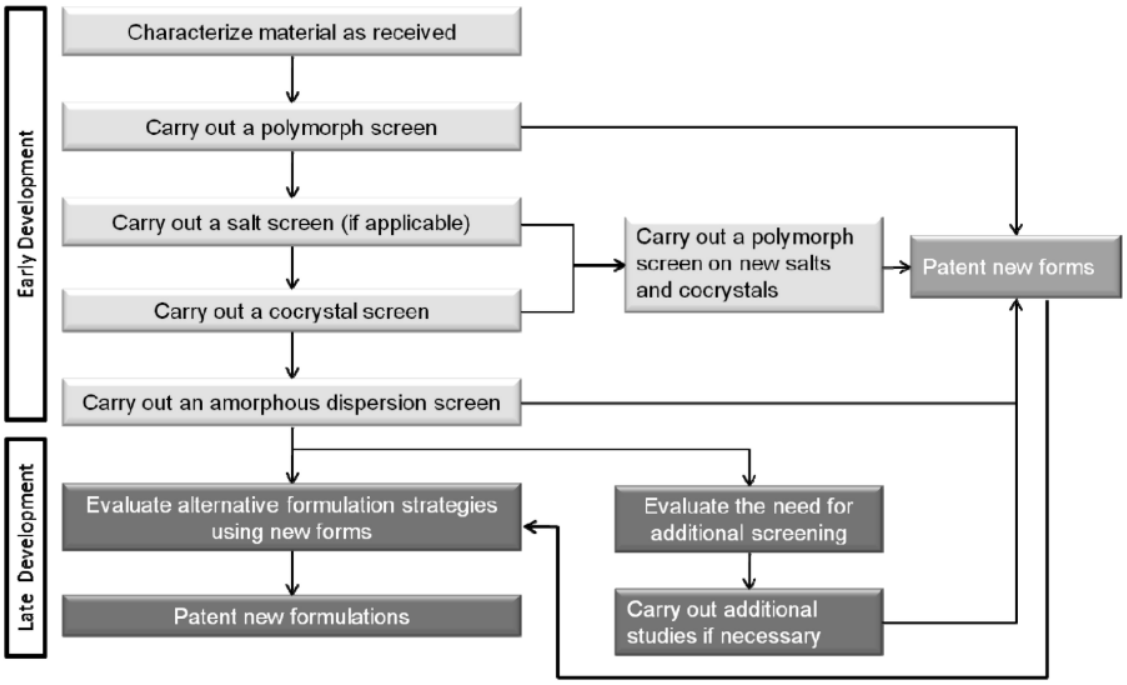

Figure 10: Screening strategies during early and late development [19]

As mentioned in the first chapter, during this thesis work New Chemical Entities (NCEs) under development for the inhalation route were studied. Due to the strict requirements of inhalation drugs related to the delivery to the lungs (Chapter 1), it is often necessary to perform crystallization studies in a very early phase of drug development, generally earlier than for other kind of drugs, such as oral drugs. So, during the late phases of drug discovery, solid form screenings are generally performed on different NCEs, frequently facing with issues such as not ideal purity and low availability of material, in order to early identify a potential developable solid form. In Figure 11 the different kind of crystallization screenings that can be performed depending on inhalation drug development phase are reported.

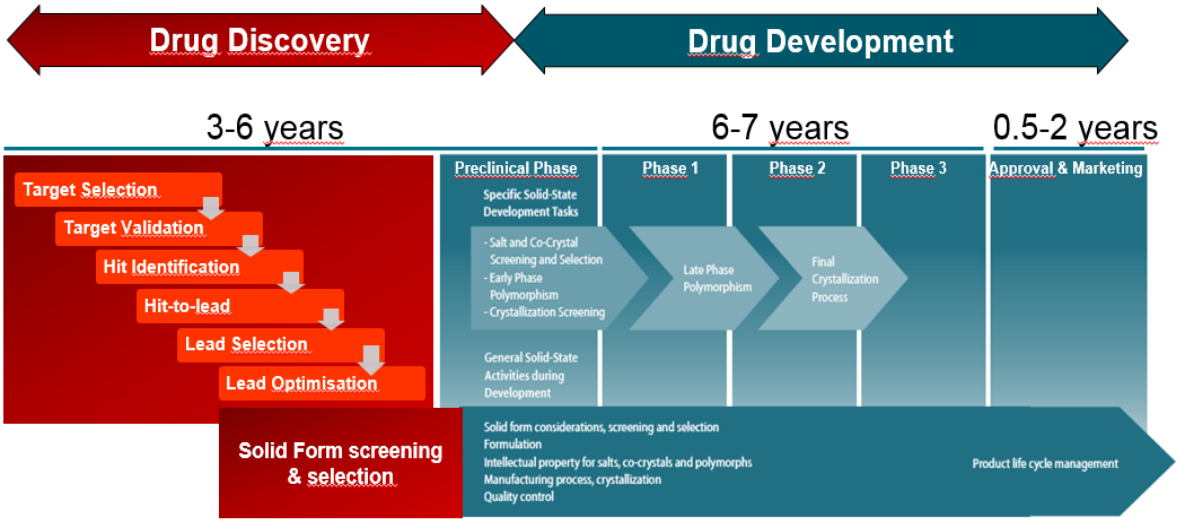

Figure 11: Crystallization techniques during drug development. Adapted from [29]

During crystallization process, different crystal forms can be obtained and they include polymorphs, solvated, hydrated, anhydrous and even amorphous materials. One goal is usually to identify the thermodynamically stable form early in the development to evaluate its properties and developability as a pharmaceutical product. Generally, fast crystallization techniques would preferentially lead to a metastable form while a slow crystallization process would favor the obtainment of a stable one. In Figure 12 this concept is illustrated, showing a summary of the main crystallization techniques used in screening experiments together with their typical time frames.

During this thesis work, and generally during the routine solid state laboratory work, some of those techniques were frequently used especially, but not only, the solvent based experiments. For example, slurring and temperature cycling experiments are widely used both in bench and mid-high throughput conditions thanks to laboratory instruments such as Polar Bear® by Cambridge Reactor Design[30] or Crystal16® by Technobis[31] (Figure 13). In fact, in the case of Crystal16®, this instrument allows to perform simultaneously different experiments, for example varying temperature or speed ramp, on 16 different samples at one time. With the Polar Bear® instrument it is possible to apply the same temperature range on until 24 samples, depending on used vials.

The screening of crystal forms affords a landscape of possible outcomes of crystallization experiments in terms of, eg, different polymorphs, solvates, hydrates. Once these forms have been obtained, a complete structural characterization is desirable, and for this reason the ideal condition would be to obtain single crystals adapt to be analyzed by Single Crystal X-Ray Diffraction (SC XRD). Therefore, during this work, other techniques such as evaporation or anti solvent diffusion were used, not only during crystallization screenings but also in order to obtain crystals suitable for SC XRD analyses.

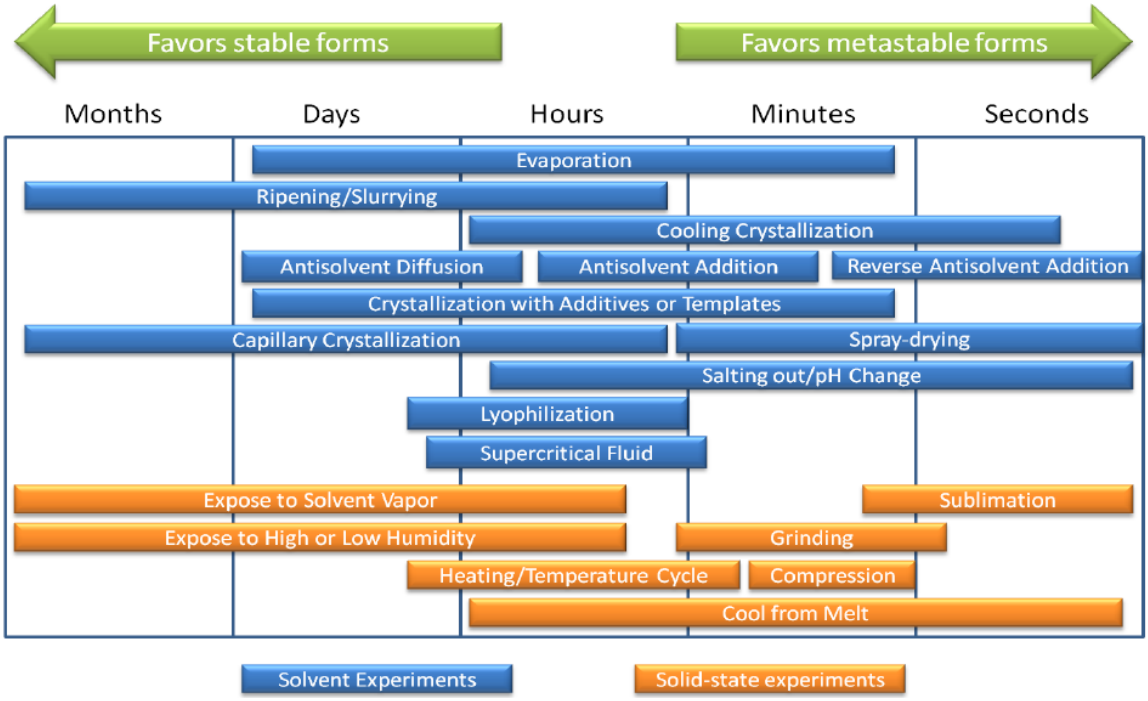

Figure 12: Time frames for common crystallization screening experiments [19]

Figure 13: Left - Polar Bear® instrument [30]; Right - Crystal16® instrument [31]

The choice of crystallization methods has an influence on which form is produced, so it is useful to perform crystallization screening using different methods. Usually, a stable form screening includes slow cooling, slow evaporation, slurring, slow anti-solvent addition or combination of those crystallization techniques.

In Table 1 a list of the “classical” crystallization methods and their degrees of freedom is reported. Many of these processes, such as slurry ripening or crystallization by cooling, are influenced not only by the chosen solvent or by the temperature, but also by the initial solid form that could be a polymorph or a solvate or an amorphous material. This can affect the solubility and hence the degree of supersaturation [25].

Method	Degrees of freedom
Crystallization by cooling a solution	Solvent or solvent mixture type, cooling profile, temperature at start, temperature at end, concentration
Evaporation	Solvent or solvent mixture type, initial concentration, evaporation rate, temperature, pressure, ambient, relative humidity, surface area of evaporation vessel
Precipitation	Solvent, anti-solvent, rate of addition, order of mixing, temperature
Vapor diffusion	Solvents, rate and extend of diffusion, temperature, concentration
Suspension equilibration (slurry ripening)	Solvent or solvent mixture type, temperature, ratio of solvent to solid, solubility, temperature programs, stirring/shaking rate, incubation time
Crystallization from the melt	Temperature programs (min, max, gradients)
Heat induced transformations	Temperature programs
Sublimation	Temperature hot side, temperature cold side, gradient, pressure, surface type
Desolvation of solvates	Temperature, pressure
Salting out	Type of salt, amount and rate of addition, temperature, solvent or solvent mixture, concentration
pH change	Temperature, rate of change, acid/base ratio, method: acid/base added as solution or in gaseous form
Lyophilization	Solvent, initial concentration, temperature and pressure programs

Table 1: List of “classical” crystallization methods

In order to identify the solid form obtained, different analytical techniques are widely used. Those analyses allow to determine information that unambiguously identify the form, such as crystalline pattern, with the XRPD analysis, and melting point with termal analysis .

During this thesis work, the following techniques were applied in order to identify the potential crystalline hits obtained:

- X-ray Powder Diffraction (XRPD) / Variable Temperature XRPD
- Differential Scanning Calorimetry (DSC)
- Polarized Light Microscopy (PLM)

Instead, other techniques were used in order to obtain a further characterization of the isolated solid form. For example:

- Thermogravimetric analysis (TGA)
- Dynamic vapor sorption (DVS)
- Moisture analysis by Karl Fisher
- Infrared and/or Raman spectroscopy
- Hot stage microscopy
- Single Crystal X-ray Diffraction (SC-XRD)
- Solid State Nuclear Magnetic Resonance (SSNMR)

In Table 2 a list of methods used for polymorphs characterization is reported and the related information given.

Type of analysis	Methods	Information given
Diffraction	XRPD	Crystallinity; crystalline form
	SC-XRD	Structural characterization; absolute configuration; presence/position of water or solvents; counter ions stoichiometry
Thermal analysis	DSC	Melting point; glass transition
	TGA	Volatile components
	Hot stage microscopy	Form changes
Moisture analysis	DVS	Water uptake; form changes
	Karl Fisher	Water content
Spectroscopy	Infrared	Interactions; crystalline form
	Raman	
	SSNMR	Mapping; imaging; crystalline form identification

Table 2: Polymorphs characterization methods

2.3. Single Crystal X-ray Diffraction

As previously discussed, especially for the inhalation field, the identification of a suitable crystalline form of an API in an early phase of drug development is crucial.

For this reason, in this thesis work a particular focus has been devoted to enhancing the use of Single Crystal X-ray Diffraction (SC XRD) for early characterization of crystal structures. In fact, traditionally SC XRD was used for final assessment of the molecular structure, due to its cost, to the need of highly experienced personnel, the need of good quality and decently sized (0.1 mm per side) single crystal. Another important factor to consider is the time of the measurements which, 20 years ago, routinely amounted to several days and it was reduced to a typical 12 hours in the last ten years. However, thanks to the latest developments in instrumentation, nowadays it is possible to collect data and solve structures in a matter of a couple of hours, and to challenge crystals with linear dimensions of the order of 10 microns.

During the three years of this work a new generation instrument equipped with a dual microfocus source (Cu and Mo) has been used, the D8 Venture® by Bruker (Figure 14). The main characteristics of this new generation instrument are the following:

- Dual IμS Microfocus Source (Mo and Cu)
    - brighter beam, up to twice the intensity of conventional X-ray microfocus sources
    - high reliability and long tube lifetimes, with very low power consumption and very little decay of intensity with time
- 4-circle kappa goniometer
    - sample-positioning freedom for the collection of a nearly infinite number of independent observations
    - the motorized detector track is automatically adjusted to the optimal detector-to-sample distance based on unit cell dimension and crystal quality
- Detector PHOTON 100
    - large 100 cm^2 active area
    - shutterless data collection for fast acquisition speed and high data quality
- APEX3 Software
    - fully integrated, intuitive and user friendly

- world-class algorithms for data acquisition and data processing

The presence of the Cu microfocus source allows the unambiguous determination of the absolute stereochemistry of chemical entities which contain first row elements only, which is very common in pharmaceutical APIs. SC XRD is in fact one of the direct method to assign the absolute configuration of a molecule, based on the anomalous dispersion phenomenon which is particularly significant for heavy atoms and long XR wavelength. With traditional diffractometers equipped with Mo radiation, the reliable determination of the configuration of stereocenters for molecules containing only first row elements is questionable, while with the microfocus dual source the experiments for assessing absolute configuration have been routinely performed. Moreover, as mentioned above, thanks to its performance the time of analysis was drastically reduced allowing measurement of crystals of average quality in less than one hour.

Figure 14: D8 Venture® by Bruker. Reprinted from [32]

Besides this new generation instrument can be used not only for structural characterization of molecules potentially developable, but also for the identification of crystalline unknown compounds in addition to more traditional techniques such as Mass Spectrometry and NMR, thanks to the rapid time of analysis and to the accurate responses. In particular, during this thesis work it was used for cases where the above techniques were unsuccessful, allowing the identification of reaction intermediates unequivocally confirming the position of halogenation reactions.

However, the most intensive use was for the characterization of the crystal structure of NCEs and to obtain all the relevant related information, such as:

- Confirmation of the presence and position of water or solvent molecules and evaluation of their role in the crystal structure (eg: understanding if the molecules were in channels, cavities or if they were strongly involved in the crystal lattice). This information has been very useful for better understanding and predicting the physical stability of the API, its handling and processability. For example, mobile molecules of water may easily lead to partial amorphisation during micronization and/or formulation as Dry Powder.
- In case of a salt, confirmation of the presence and position of counter-ions and their role in the crystal structure. The understanding of the favourite interactions of the molecule with counter-ions (or co-formers) was a precious aid in better designing salt/co crystal screenings.

During this thesis work an increased number of molecules of industrial interest has been analyzed using the D8 Venture®. In fact, the structural characterization of at least 15 molecules has been determined, allowing, for example, to better understand the presence and role of water molecules, in case of hydrate molecules. The information obtained has revealed to be very useful to better understand and predict some physicochemical properties of the molecules thus allowing a wider comprehension of their developability.

2.4. Crystallization and crystal growth

Growing a suitable single crystal is the most decisive step of a successfully single crystal X-ray structure determination [33].

Preferably grown from a homogeneous solution, crystals form when the molecular units pack together based on interactions and repulsions among them. When packing happens slowly, the molecules will fit together until a crystal, a three-dimensional repeating unit, is formed. According to the Classical Nucleation Theory, crystallization occurs in two steps: first, crystal nuclei of critical size are formed; second, some of these nuclei grow into mature crystals [34]. Nucleation occurs only when a threshold level of supersaturation is reached (Figure 15). Once nucleation has been initiated, critical nuclei are ready to evolve into mature crystals by the growth process.

The crystal growth process consists of several stages through the growth unit (the critical elements of how a specific molecular species has assembled in a crystalline state in three dimensions) [28]. These stages include:

- Transport of a growth unit, by convention and diffusion, from or through the bulk solution to a site on the crystal face, which is not necessarily the final growth site
- Adsorption of the growth unit at the site
- Diffusion of the growth units from the impingement site to a growth site
- Incorporation into the crystal lattice

There are several techniques used for triggering crystallization, including vapor diffusion, evaporation, solvent layering; in the following paragraphs some of the most frequently used are listed and described.

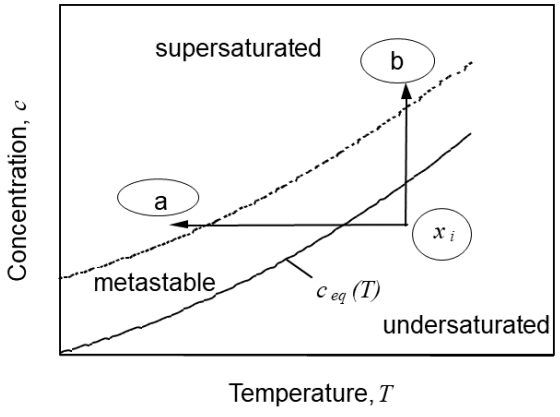

Figure 15: Generic solubility curve, showing the process of reaching supersaturation by an initial solution of composition X by (a) cooling and (b) evaporation.

2.4.1. Slow cooling

Slow cooling is a crystallization technique good for less soluble solute-solvent systems where the boiling point of the solvent is in the range 30–90°C. The procedure consists in preparing a saturated solution where the solvent is heated to just below the boiling point, then gradually decrease the temperature and leave the sample at lower temperature for several days (Figure 15 case (a)). This best applies if the solubility of the API changes drastically with temperature variations (Figure 16).

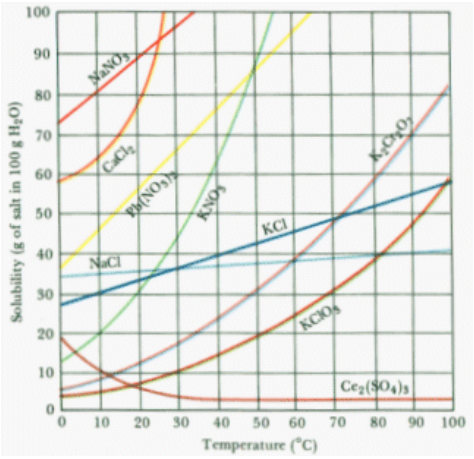

Figure 16: Solubility as a function of temperature for different inorganic salts. Reprinted from [35]

2.4.2. Solvent evaporation

Solvent evaporation is the simplest crystallization technique for air stable compound. The procedure consists in the preparing a near saturated solution in a suitable solvent

or solvent mixture. The sample can then be left for several days in a sample vial that has a perforated cap (the size of the perforations is an experimental variable) to allow solvent slow evaporation (Figure 15, case (b)).

2.4.3. Vapor diffusion

Vapor diffusion occurs in a closed system: a small vial with a near-saturated solution of the sample in solvent A is placed into a larger outer vial containing anti-solvent B. As the solvents evaporate, solvent B vapor diffuses into solvent A in the solution, which gets richer and richer in the anti-solvent B, and the solubility of the API decreases. So the process will decrease the solubility of the compound in solution enough so that crystals will slowly form and, if the rate of evaporation is slow, molecules can pack slowly diffusing into the inner solution.

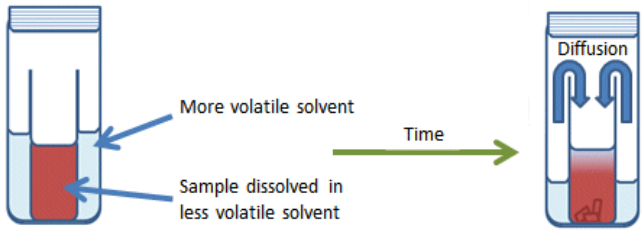

Figure 17: Vapor diffusion method. Reprinted from [36]

Solvent B is chosen to mix well with solvent A and has a lower temperature of vaporization than solvent A.

2.4.4. Gel crystallization

Gels are used as growth media for a wide range of compounds, including both inorganic and organic compounds and proteins, because they provide a diffusive medium for the mass transport of molecules during crystallization and eliminate convection and sedimentation resulting in higher crystal quality [37].

Even if not frequently, gels can be also used during polymorph screenings to provide different nucleation mechanism from that found in solutions [19]. Moreover, the API supersaturation levels could be different in different gel matrices influencing crystal form and particle size.

This technique is widely used for crystallization of insoluble compounds. One of the drawbacks of using gels is the isolation of crystals obtained: in fact, in most of the cases, it can be only done manually removing crystal by hand and then washing the residual gel.

Basing on needs, there are three different experiment types that can be performed:

- Precipitation of a compound: generally performed in vertical tubes, the compound is solubilized with the gel in the same solvent. When the gelification occurs, an antisolvent is added in the upper part of the tube. Crystallization occurs by lowering the compound solubility. To obtain better crystals, the antisolvent should be the worst solvent possible for the target compound.

$$A_{solution} + \text{antisolvent} \rightarrow A_{crystals}$$

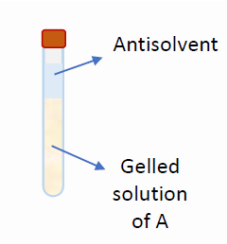

Figure 18: Gel crystallization technique: precipitation compound process

- Reaction-crystallization process: for this technique particular U-tubes are generally used (Figure 19). Two different reagents (two co-formers or compound - counter ions) are solubilized with gel in the same solvent. The controlled counter-diffusion of the two reagents in a selected pure solvent yields to the

desired products; the product solubility in the final solution must be very low to obtain good crystals.

$$A_{solution} + B_{solution} \rightarrow C_{crystals}$$

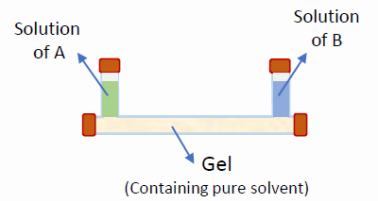

Figure 19: Gel crystallization technique: reaction-crystallization process

2.5. Vapor diffusion in gel: the same principle of the traditional vapor diffusion technique previously described is applied, but the process is slower and improved due to the presence of gel.

Moreover, there are different gel types that can be used, as:

- Agarose
- Poly (ethylene oxide) (PEO)
- Silica (from Na_2SiO_3)
- Silica (from $Si(CH_3O)_4$)

Usually, the determining criteria to select the gel type are the solvent and the preparation condition of the gel. For example, to prepare agarose gel, soluble only in water, heating at about 85°C is required, therefore thermally labile compounds cannot be crystallized in agarose gel.

Instead, poly (ethylene oxide) is soluble in a wide range of solvent, so its application is compatible with a range of molecules soluble in different solvents. In Table 3 is reported a list of solvents where PEO is totally/partially soluble or insoluble. In the PEO columns, G indicates that a gel is formed, F that a flocculate is formed and I that the PEO is insoluble [37].

Group 1		Group 2		Group 3	
Solvent	PEO	Solvent	PEO	Solvent	PEO
Acetonitrile	G	Acetone	F	Diethyl Ether	I
Benzene	G	Butanone	F	Diisopropyl Ether	I
Chlorobenzene	G	*n*-Butanol	F	Heptane	I
Chloroform	G	Ethanol	F	*n*-Hexane	I
1,2-Dichloroethane	G	Ethyl Acetate	F	Isooctane	I
Dichlorometane	G	2-Propanol	F	Octane	I
Nitrometane	G	Methyl Acetate	F	*n*-Pentane	I
Water	G	Methanol	F		
		1-Propanol	F		
		Tetrahydrofuran	F		
		Toluene	F		

Table 3: Solvent list for PEO preparation

Polymorphism of Active Pharmaceutical Ingredients

3. Polymorphism of Active Pharmaceutical Ingredients

3.1. Definitions

3.1.1. Polymorphism

Polymorphism is defined as the ability of a substance to exhibit different crystal structures having the same chemical composition, but characterized by different molecular arrangements or different molecular conformations [38]. It must be stressed that sometimes in the pharmaceutical jargon hydrates, hydrochlorides, and in general multiple modifications of a crystal form are referred to as polymorphs, or pseudopolymorphs, but there is nowadays a general and strong consensus that the addition of other components in the crystal structure does not lead to polymorphs: depending on the nature of the added component, it leads to salts, solvates or co crystals [39]. Polymorphs exhibit different physical-chemical properties, and can be patented separately [26].

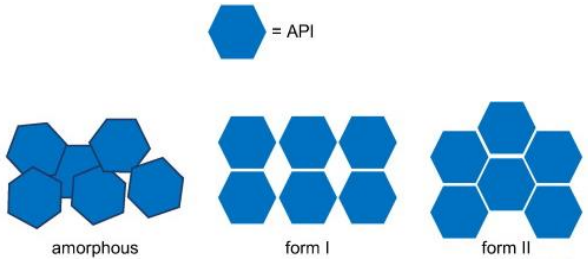

Figure 20: Polymorphs of a generic substance [40]

3.1.2. Salts

A salt is formed when a molecule is combined with an acid or a base and proton transfer occurs so it is made up of two charged species. A salt has a different composition from the neutral molecule and is therefore a different chemical entity, generally with different properties like solubility and chemical and pH stability. Obviously, a salt will also potentially exhibit polymorphism (Figure 21).

Generally, the pK_a difference between the API and the co-former can be used to predict whether salt formation has occurred or not. Food and Drug Administration (FDA) Regulatory guidelines suggest that proton transfer is expected when the difference in the pK_a (pK_a(acid) - pK_a(base)) is greater or equal to four [41].

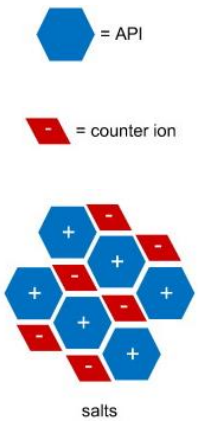

Figure 21: Polymorphs of a salt [40]

3.1.3. Solvates and co crystals

When an additional neutral molecule is part of the crystalline structure of a substance and no proton transfer occurs, there could be different forms depending on the state of the pure co-former at room temperature. For example, when the co-former is the solvent of crystallization, the resulting species is called a solvate, while when the co-former is solid at room temperature, the resulting species is called a co crystal. There are different

definitions of co crystals but, nowadays it is assumed that co crystals are formed by two or more components that form stable solid aggregates on their own at room temperature [42].

As for salts, solvates and co crystals can also exhibit polymorphism (Figure 22).

The FDA Regulatory guidelines, in trying to draw a line between salts and co crystals, suggest that when the differences in the pK_a (pK_a(acid) - pK_a(base)) is less than four a co crystal is expected [41;43].

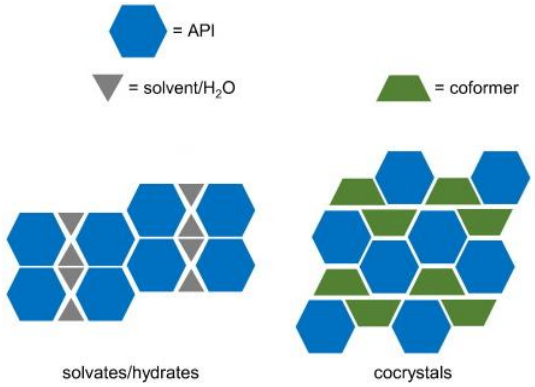

Figure 22: Polymorphs of solvates/hydrates and co crystals [40]

When the solvent, which takes part in the crystalline structure, is water the subgroup of solvates is called hydrates. Hydrates are of particular interest in the pharmaceutical industry because water is non-toxic and acceptable in the formulated products.

Even though the definitions are theoretically quite clear and simple, a crystal structure can contain more than one co-former, resulting in, for example, solvates of a co crystal, hydrates of a salt or hydrated solvates. Moreover, all of these species can obviously exhibit polymorphism and crystallize in more than one structure with the same chemical formula.

The growing importance of controlling the landscape of crystal forms accessible to an API is due to the fact that there is an observable trend that new drug entities are becoming larger and less soluble, less absorbable and bioavailable. As a consequence, major efforts are required by the pharmaceutical industry to develop and

market an active drug which can be delivered to the body in a suitable crystal form [44]. For this reason, the selection of a salt form remains a helpful and widely used tool,but solvates and hydrates production could be considered as an additional tool to develop APIs with enhanced properties, especially solubility.

3.2. Role of polymorphism in drug properties

Solid state properties, including polymorphism, can have a deep impact on many aspects of the manufacturing, handling and formulation of a drug product or substance. Although identical in chemical composition, polymorphs can have very different properties such as bioavailability, solubility, hygroscopicity, melting point, stickiness, bulk density, dissolution rate, stability (both chemical and physical), flowability, colour, compactability and crystal habit [55].

The knowledge of the properties of the different solid forms of an API can therefore lead to the choice of the form with the most suitable characteristics, leading to a better bioavailability, longer shelf-life, easier formulation or more robust process control.

3.2.1. Biopharmaceutics Classification System

In order to correctly understand and assess the impact of solubility on bioavailability of a drug product, a useful tool is the Biopharmaceutics Classification System (BCS). This is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability [45]. Solubility is an essential property of drugs, because they must dissolve in order to be absorbed through membranes and reach the site of action. Consequently, solubility is one of the most critical and important parameter influencing drug bioavailability, that is, the ability of a drug to be available in an appropriate concentration at the site of action, independently of the pharmaceutical dosage form and route of administration. It is widely used for regulatory purposes to help establish bioavailability and bioequivalence, but can also give information regarding which properties can limit bioavailability for a specific substance [46].

According to BCS, drug substances or APIs are divided into high/low solubility and permeability classes as follows (Figure 23):

- Class I : High Solubility - High Permeability
- Class II : Low Solubility - High Permeability
- Class III : High Solubility - Low Permeability
- Class IV : Low Solubility - Low Permeability

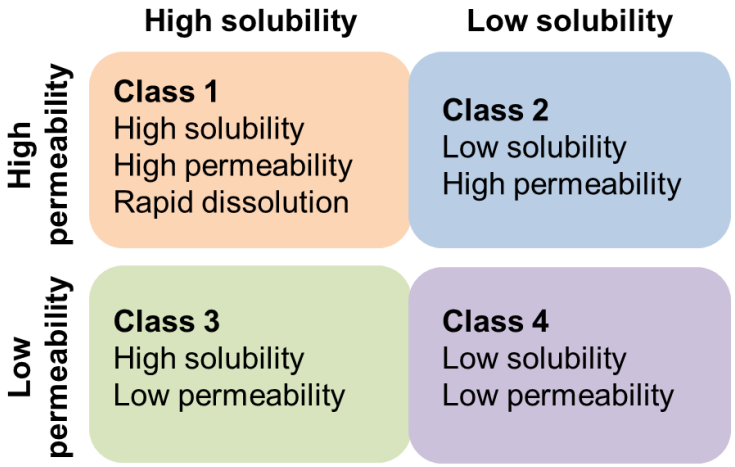

Figure 23: Biopharmaceutical Classification System. Adapted from [47]

Complementary to BCS, there is the Biopharmaceutics Drug Disposition Classification System (BDDCS), proposed by Wu and Bennet in 2005 with the purpose to predict drug disposition and potential drug-drug interactions in the intestine and the liver, and potentially the kidney and brain, taking into account the role of transporters [47]. In Figure 24 the classification of drug and New Molecular Entities (NMEs) based on BDDCS is reported.

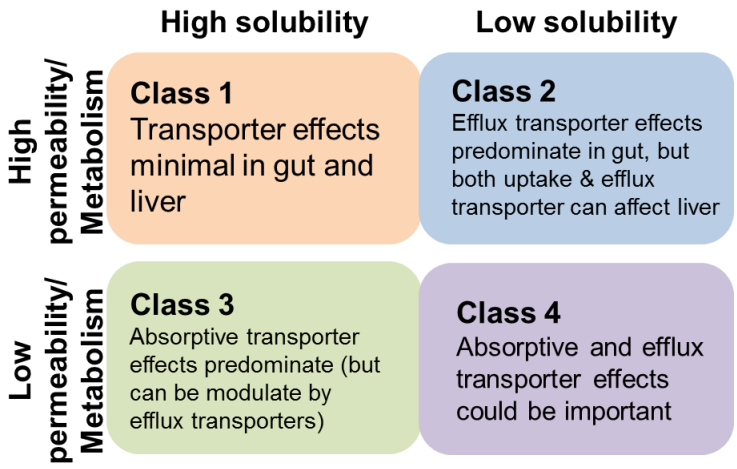

Figure 24: Biopharmaceutical Drug Disposition Classification System. Adapted from [47]

The BCS and BDDCS are important when dealing with different solid forms of pharmaceutical substances because they give indication as to whether or not polymorphism has an impact on the bioavailability of the formulated product. For instance, where solubility is low, for example in classes II and IV, polymorphism can be an issue in the bioavailability of the formulated drug. Numerous strategies exist for enhancing bioavailability of drugs with low aqueous solubility, such as preparation of co crystals, metastable polymorphs, high-energy amorphous forms or ultrafine particles [23]. Developing amorphous materials could present important challenges because of its potential conversion to a crystal form with a significantly reduced bioavailability. While, a compound that shows best bioavailability with a crystalline material can have important advantages in its developability [48].

In Chapter 4 co crystals and their application in pharmaceutical field will be deeply discussed.

3.2.2. Thermodynamics of polymorphs

In the previous chapters, the importance of obtaining a thermodynamic stable solid form has been discussed. As mentioned before, the most thermodynamically stable forms generally have the lowest solubility, therefore the lowest bioavailability [49]. In some cases, for example for very insoluble APIs, the chosen form might not be the most stable but the most soluble, being the better or the only form allowing to reach the desired human dose. In these cases the clear understanding of the existing environment of the latter form is crucial for ensuring the developability of the drug. In fact, knowledge of the polymorphic behavior plays a relevant role in the pharmaceutical industry to ensure that the final product contains the desired polymorph and to avoid appearance or disappearance of polymorphs during process development. There are well-documented cases of crystal forms that were observed over a period of time but not thereafter, having been apparently displaced by a more stable polymorph [50]. One well known example of disappearing polymorphs is the case of Ritonavir. Ritonavir is an antiretroviral medication used to treat HIV/AIDS and it was originally marketed as an ordinary capsule, which did not require refrigeration, as a crystal now called Form I. However, Ritonavir exhibits polymorphism and, in this case, the solubility and the bioavailability appear very different in the two polymorphs. During development, only the Form I was found, but later, a more stable polymorph (Form II), with lower free energy, appeared. This more stable, and so less soluble, crystal form compromised the oral bioavailability of the drug. Even a trace of Form II can catalyze the transformation from Form I, the more bioavailable, to Form II, the more stable. Form II, which was poor soluble and hence doesn't have therapeutically effect, entered production lines. The capsules have been replaced with refrigerated gelcaps, to solve the crystallization problem of the original capsules [51].

In order to better understand this concept, it is useful to describe the differences between a monotropic and an enantiotropic system. A monotropic system is a system in which one form is more stable than the other at temperatures below the melting points, while in an enantiotropic system there is a transition temperature below the melting points [52]. In other words, in the latter case one form is stable below the transition temperature, and the other form is stable above the transition temperature. In an enantiotropic system, the metastable form at room temperature can be obtained by heating the stable form above the transition temperature. In a monotropic system, the stable form at room temperature can be

obtained by heating the metastable form at any temperature. The rate of transformation can be facilitated by heating the metastable form at high temperature [53]. So, for example, if a metastable form of a monotropic system is desired there could be applied precautions to avoid transformation from a metastable to a stable form. Otherwise, no precautions are needed if the desired form in a monotropic system is the stable one, while information about the transition point are necessary to obtain and maintain the desired polymorph in an enantiotropic system [25; 54].

In Figure 25, the energy/temperature diagrams of dimorphic systems are reported.

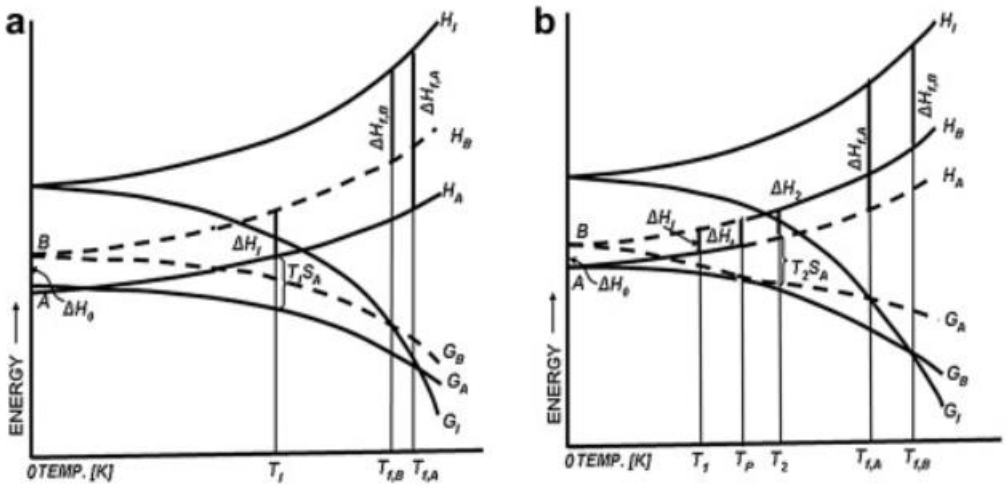

Figure 25: The energy/temperature diagrams of dimorphic systems are reported. (a) monotropic systems, (b) enantiotropic systems (Tp: transition point; Tf: fusion point; H: molar enthalpy; G: molar free energy; S: molar entropy; A, B: crystalline modifications; l; liquid phase). Reprinted from [54].

Each solid phase of a compound has a characteristic value for its physicochemical properties, including thermodynamic values, which allows to understand and predict the influence of environmental conditions, such as temperature, pressure or relative humidity, on the nature of each polymorph and on the tendency of one phase to transform into the another [25]. Different crystal forms may also exhibit different

compressibility or hygroscopicity, which is also a very important property to take into account because it can have an effect on the stability of the formulated product.

Furthermore, other characteristics have to be taken into account as the crystal habit, which may depend on crystal form, and that can play a crucial role in the ease of handling and processability of a pharmaceutical powder. Crystal habit, on the other hand, might be also influenced by kinetics conditions during crystal growth, which usually occurs out of thermodynamic equilibrium.

3.2.3. Crystallography of polymorphs

As mentioned in the previous chapters, crystalline solids consist of a regular three-dimensional packing of its constituent atoms and molecules, while amorphous solids only show atomic organization at short distance. Polymorphism may be defined as the existence of different molecular packings of the same molecules and it has become of relevant importance for the pharmaceutical industries, also for regulatory reasons [25].

X-ray crystallography has evolved as a method available to determine the molecular structure at atomic resolution which is a helpful prerequisite for rational drug-design and structure-based functional studies [16]. Demonstration of a non-equivalent structure by Single Crystal or Powder X-ray Diffraction is currently regarded as the definitive evidence of polymorphism [55].

In fact, many solids occur as microcrystalline powders and, as mentioned in Paragraph 2.3, in these cases growing single crystals could be very challenging. X-ray powder diffraction (XRPD) is one of the most reliable techniques for polymorph differentiation which yields a fingerprint of a solid phase with numerous peaks whose positions correspond to periodic spacing of atoms in the solid state. Generally, different lattice constants rise to different peak position, as illustrated in Figure 26 where there is a comparison of four polymorphs of carbamazepine [38].

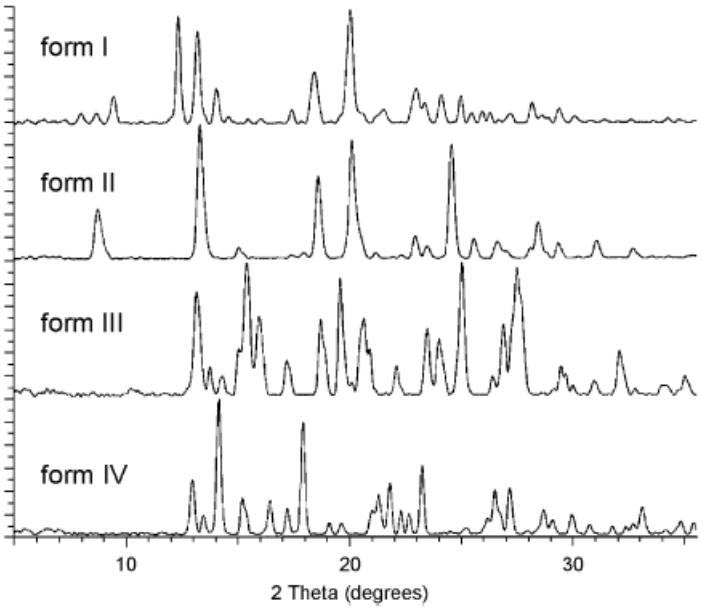

Figure 26: XRPD comparison of the four polymorphs of carbamazepine. Reprinted from [38].

Furthermore, XRPD has found numerous applications in pharmaceutical field such as characterization of intermediates of solid state reactions products. In addition, this technique can play an important role on evaluation of form stability and possible phase conversions during manufacturing and formulation developing.

Generally, over the years, X-ray crystallography has constantly increased its application fields (Figure 26).

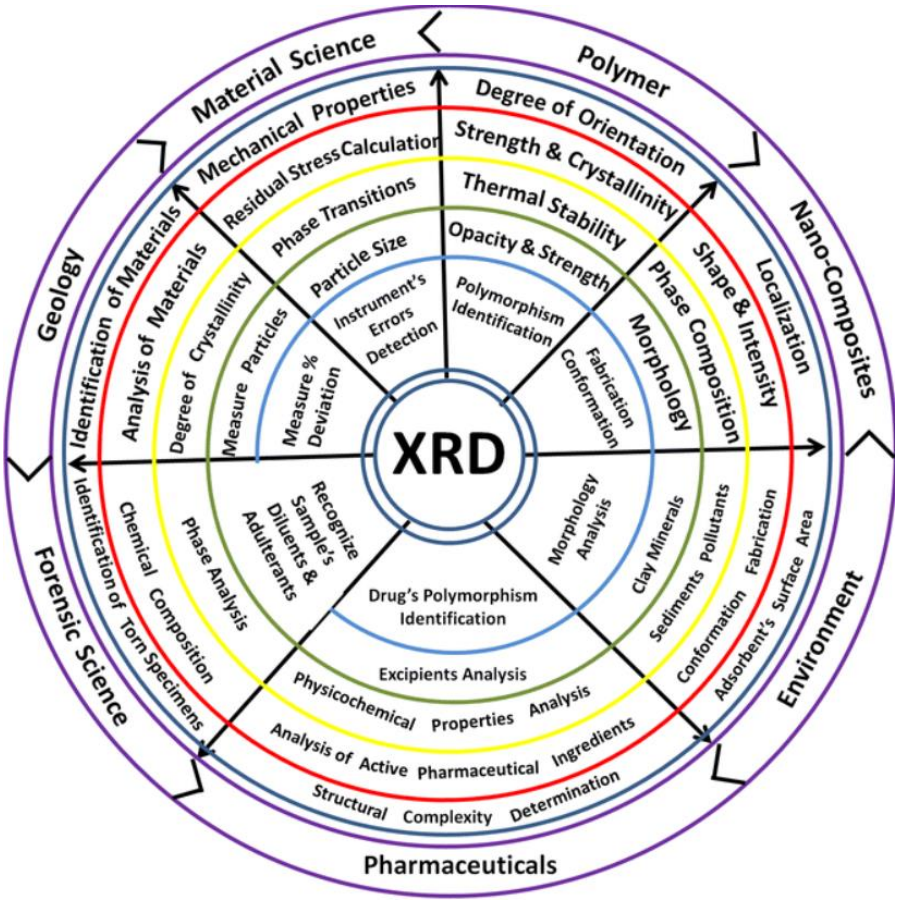

Figure 26: Analytical applications of X-ray diffraction in different fields. Reprinted from [56]

3.2.4. Polymorphism and patents

Polymorphism presents challenging issues to patent systems [57]. A major role in the importance of solid in the pharmaceutical industry is played by patentability of solid forms and the protection that this can offer.

According to the Oxford English Dictionary definition, a patent is “a license to manufacture, sell, or deal in an article or commodity, to the exclusion of other persons; in modern times, a grant from the government to a person or persons conferring for a certain definitive time the exclusive privilege of making, using, or selling some new

invention.” The two main patent systems when dealing with polymorphism of APIs generally are considered to be the EPO (European Patent Organization) and the USPTO (United States Patent and Trademark Office), as these cover the main global markets for pharmaceuticals. As reported by EPO guidelines, among the requirements needed to obtain a patent of a new polymorphic form [58]. there are novelty and non-obviousness. By definition a new crystal form is novel. Since crystal forms cannot be predicted a priori they are also not obvious [59].

As seen in the other chapters, a particular crystalline modification can lead to important chemical, physical or also biological advantages. For this reason, the discovery or the preparation technique of a new crystal modification can represent an opportunity to claim an invention that can be potentially recognized in a patent. So, for industries, and for pharmaceutical industry too, granting and maintaining the patent exclusivity has become crucial, with considerable economic consequences. This applies, not only of the chemical formula of a NCE but also of information related to polymorphism and solid state properties of the solid phases.

Co crystals and their role in pharmaceutical science

4. Co crystals and their role in pharmaceutical science

The range of crystalline forms that are available for an Active Pharmaceutical Compound (API) has traditionally been limited to polymorphs, salts and hydrates or solvates. As discussed in the previous chapters, developing and delivering a crystalline form of an API as solid dosage is becoming more convenient and important for clinical, legal and regulatory perspective. Listed are some of those reasons:

- Crystalline forms are more stable and reproducible and purification steps are more easy with a crystalline than an amorphous form
- The intrinsic solubility and the dissolution rate of different crystal forms are variable and this has a strong influence on bioavailability
- The unpredictability of crystal structures, and their related physical properties, is strongly related to the possibility of obtaining and maintaining patent protection for an API [60]

In this contest, pharmaceutical cocrystals became more and more attractive for pharmaceutical scientists because of the potential diversification of crystal forms for an API and, moreover, because they can lead to important improvements in physicochemical and pharmacokinetics properties of the compounds, such as bioavailability, solubility and dissolution rate, tableting, melting point, permeability [61]. Moreover, cocrystals expand the range of solid forms available for formulation.

Often, cocrystals offer the opportunity to transform an amorphous or a hard to crystallize API in an easily handleable and solid crystalline form. As mentioned before, cocrystals formation can help to solubilize a poorly soluble API, by means of what has been called the 'spring and parachute effect' [61; 62]. It occurs that the solution content of the API, as soon as the cocrystal dissolve, gets higher than the thermodynamic limit for the insoluble API; if the drug is absorbed during the initial time, its bioavailability is increased. After a time lapse in fact the substance can undergo a recrystallization to the most stable pure API form when in contact with the solvent [63].

Moreover, discovery or design a new cocrystal offers new opportunities for the exploitation of intellectual properties.

4.1. What is a cocrystal

A cocrystal is a multicomponent crystal containing two or more molecular components in stoichiometric ratio. These molecules are assembled from specific non-covalent interactions, such as hydrogen bonds, ionic, van der Waals and $\pi - \pi$ interactions [52] . Recently the class of ionic co crystals has been defined, whereby one of the components is a salt [26].

However, there is a practical difference between cocrystals that include specific and intentionally designed coformers and those that have been accidentally included in the system, for example because they are potential crystallization solvents. So, there was proposed that, to be correctly defined cocrystal, none of the components of the cocrystal system should have been a solvent used during the crystallization process (Figure 28) [63].

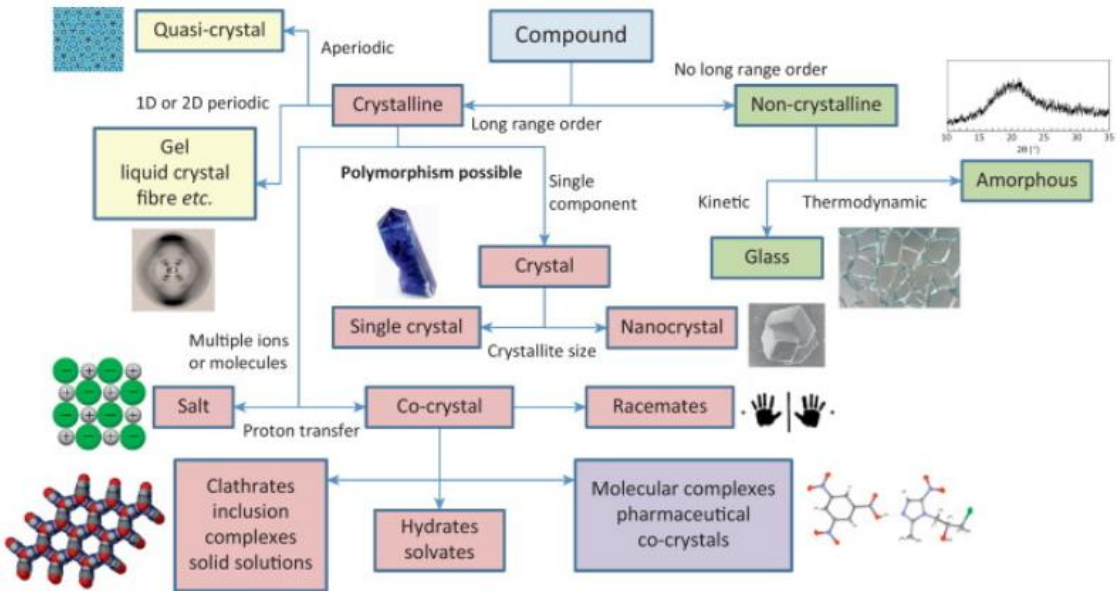

Figure 28: Classification of solid forms. Reprinted from [63]

Pharmaceutical cocrystal belong to the sub-class of multicomponent system in which at least one of the component is an API and the coformer is selected from the Generally Recognized as Safe (GRAS) list of substances [64] that have to be non-toxic with no adverse side effects. Despite of the strict definition of cocrystal, cocrystals of pharmaceutical compounds with water, solvents or other solid coformers, are conceptually indistinguishable from one another.

As mentioned before, different physicochemical properties of a crystalline solid could be enhanced by the use of cocrystals. In Figure 29 a list of examples of pharmaceutical cocrystals systems is reported, with the corresponding improved property.

API	Co-crystal former	Preparation method	Enhanced property (if reported)	Reference
Aspirin	4,4'-Dipyridil	Slurry conversion		Walsh et al 2003
Caffeine	Oxalic acid Glutaric acid	Solvent-assisted grinding	Physical stability	Trask et al 2005
Carbamazepine	Nicotinamide Saccharin	Cooling crystallization	Physical stability, dissolution rate and oral bioavailability	Hickey et al 2007
Fluoxetine hydrochloride	Benzoic acid Succinic acid Fumaric acid	Solvent evaporation	Intrinsic dissolution rate	Childs et al., 2004
Flurbiprofen	4,4-Dipyridyl	Solvent evaporation		Oberoi et al 2005
Ibuprofen	4,4-Dipyridyl Nicotinamide	Solvent evaporation	Solubility	Walsh et al 2003; Oberoi et al 2005
Indomethacin	Saccharin	Solvent evaporation or solvent-assisted grinding	Physical stability and dissolution rate	Basavoju et al 2008
Itraconazole	Malic acid Tartaric acid Succinic acid	Solvent evaporation	Improved dissolution rate	Remenar et al 2003
Norfloxacin	Isonicotinamide Succinic acid Malonic acid Maleic acid	Solvent evaporation	Solubility	Basavoju et al 2006
Paracetamol	4,4-Dipyridyl	Solvent evaporation		Oswald et al 2004
Piroxicam	Saccharin	Solvent evaporation		Childs et al 2007

Figure 29: examples of pharmaceutical cocrystals systems reported in the literature. Reprinted from [65]

4.1.1. Mechanical properties

Mechanical properties of drugs are important for bulk powder compaction and tableting, and these properties are strongly related to the crystal structure of the compounds. The traditional mechanical deformation mechanisms of solids include elastic, plastic, viscoelastic and fragmentation mechanisms. For example, the elasticity of a solid material is its ability to undergo reversible deformation under an externally applied stress.

It is recognized by different studies that cocrystallization of drug powders is an important improvement at the preformulation stage to control the crystal packaging and to modulate the compaction and the tabletabilty of the powder [61]. One example, here just mentioned, is the study on a model of methyl gallate cocrystals with caffeine and theophylline [66]. The effect of cocrystal packing on mechanical properties and the correlation between crystal dislocation and plasticity were studied resulting in the better powder compaction of the 1:1

cocrystal of caffeine and theophylline with methyl gallarate due to slip planes in the cocrystal structure.

4.1.2. Modulating permeability

Permeability across the biological membrane is a key parameter for drug adsorption and distribution. It is classified by the estimated *n*-octanol/water partition coefficient using both Log P and Log C for the uncharged form of drug molecules. As described in paragraph 3.2.1, drug candidates which exhibit low solubility/permeability are classified as BCS class IV drugs. Generally, poor permeable drugs cause significant problems on absorption and distribution across the gastrointestinal mucosa throughout the body.

Also for this property, there are numerous studies reporting the ability of cocrystals to improve permeability of poor permeable drugs. One example is the bronchodilatator drug theophylline (THP) cocrystals with isomeric aminobenzoic acids. As reported in Figure 30, theophylline cocrystals at various stoichiometry ratio exhibit higher flux/permeability that is attributed to coformer solubility [61].

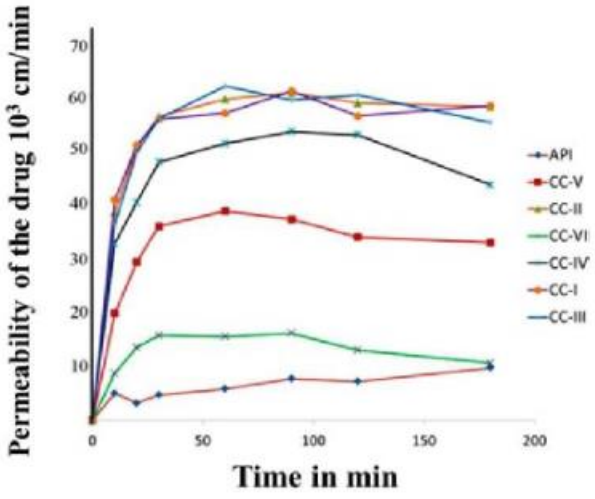

Figure 30: Membrane permeability enhanced with the use of cocrystals at different stoichiometry. Reprinted from [61].

4.1.3. Bioavailability, solubility and dissolution rate

As discussed in the previous chapters, drug molecules with limited aqueous solubility are becoming increasingly prevalent in the research and development portfolios of pharmaceutical companies. Molecules of this type can provide a number of challenges in pharmaceutical development and may potentially lead to slow dissolution in biological fluids, insufficient and inconsistent systemic exposure and consequently sub-optimal efficacy [**Errore. Il segnalibro non è definito.**]. Different strategies could be adopted in order to enhance solubility and bioavailability of this kind of drugs, and crystal engineering offers the opportunity to design and synthetize cocrystal with high aqueous solubility and oral bioavailability.

One example is from the study of the dissolution of itraconazole with succinic acid, malic acid and tartaric acid that was compared to that of pure crystalline and amorphous drugs [67]. The cocrystals behaved in a similar manner to the amorphous form, while they achieved and sustained from 4- to 20-fold higher concentration on dissolution tests compared to the pure crystalline form of the drug.

4.2. General design strategies for cocrystallization

Cocrystal screening is a process similar to polymorph and salt screening. Once an API has been selected for cocrystallization studies, a pharmaceutically acceptable coformer, non-toxic and with no adverse effects, should be chosen. Furthermore, the cocrystal formation may be rationalized by considering the hydrogen bond donors and acceptors involved. The following guidelines were proposed by Margaret Etter to facilitate the design of hydrogen bonded solids [68]:

- All good proton donors and acceptors are used in hydrogen bonding
- Six-membered ring intramolecular hydrogen bonds form in preference to intermolecular hydrogen bonds
- The best proton donor and acceptor remaining after intramolecular hydrogen-bond formation will form intermolecular hydrogen bonds to one another (but not all acceptors will necessarily interact with donors).

After the selection of coformers, there are various common cocrystallization strategies, listed below:

- use of an excess of one of the coformers with a consequent reduction in the solubility of the cocrystal in the presence of the excess component;
- slurry crystallization;
- wet milling of the solid components in the presence of just a few drops of solvents;
- involving an intermediate phase, such as a hydrate or amorphous form as part of a solid state synthesis;
- use of a metastable polymorph to give an unstable intermediate that can trigger cocrystal growth;
- seeding solutions using cocrystal seeds derived from melt crystallization using hot stage microscopy.

In Figure 31 an example of a microscopic image of a cocrystal obtained by melt crystallization with hot stage microscopy is reported [69].

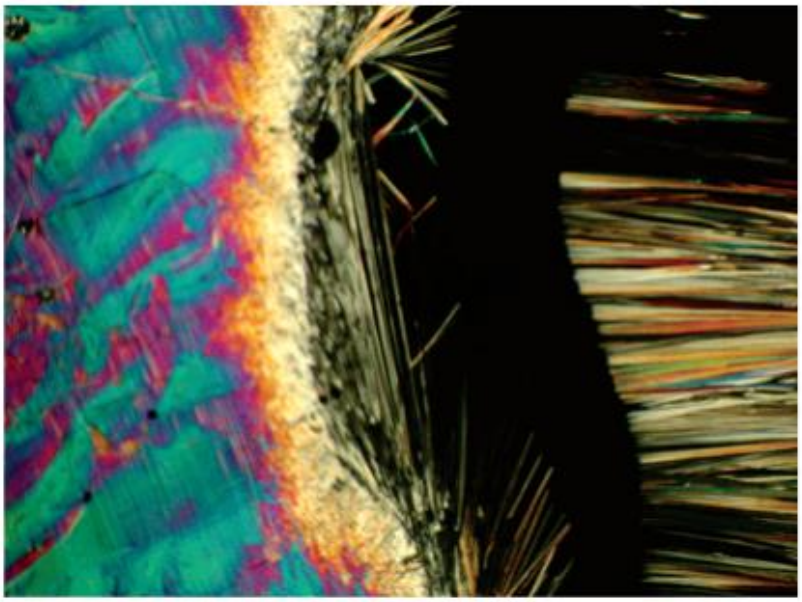

Figure 31: An optical microphotograph of a cocrystal formed using the contact method. Reprinted from [69]

However, the ability to predict *a priori* which compounds are likely to form cocrystals with a given API would provide a complementary tool to experimental screening. One approach is to analyse the structures of crystalline solids based on the pairing of H-bond donors and acceptors that form repeating H-bonded supramolecular motifs called synthons. There are two categories of supramolecular synthon: homosynthons that are formed by two identical functional groups and heterosynthons that are formed by different and complementary functional groups. Statistical studies of X-ray crystal structures in the Cambridge Structural Database (CSD) have identified commonly occurring H-bonding motifs, and these have been used to design cocrystals [70]. An approach ranking the best pairing of maxima and minima of the Molecular Electrostatic Potential (MEP), in line with Etter's principles, has also been suggested [70]. However, these methods provide only loose guidelines, and no reliable technique to assess the propensity to cocrystallization between two molecules is available so far.

Analytical techniques to characterize cocrystals are the same mentioned in Chapter 2 for characterization of polymorphs. So for the determination of the obtained cocrystals hits Powder X-ray diffraction (XRPD), thermal analyses such as Differential Scanning Calorimetry (DSC) or Thermogravimetic Analysis (TGA) and Polarized Light Microscopy (PLM) are widely applied. As described for polymorphs characterization, also for cocrystals the Single Crystal X-ray Diffraction (SC XRD) is the "gold standard" method for the structural characterization, not only because this technique allows to determine the crystal form but also because it allows the elucidation of supramolecular synthons [63].

4.3. Case studies of pharmaceutical cocrystals

The first examples of cocrystals in the contest of APIs are from 1950s when there were studied complex formation between macromolecules and pharmaceuticals such as polyvinylpirrolidone (PVP), chloramphenicol, mandelic acid, caffeine, theophylline [71,72]. However, these would not be classified as pharmaceutical cocrystals according to the current criteria.

There are several case studies that involve the formation of cocrystals with altered physical and clinical relevant properties.

4.3.1. Pharmaceutical cocrystals of fluoxetine hydrochloride (Prozac®)

The availability and marketability of a variety of APIs as chloride salts are well recognized. An approach to use such chloride salts, specifically fluoxetine hydrochloride, to generate cocrystals has been reported [73]. Fluoxetine hydrochloride is the active pharmaceutical ingredient found in the commercial antidepressant drug called Prozac®. Fluorexitine hydrochloride was cocrystallized with benzoic acid in ratio 1:1, with succinic acid in ratio 2:1, and with fumaric acid in ratio 2:1 by traditional evaporation techniques. For all the three cocrystals, the carboxylic acid was found to form hydrogen bond to the chloride ion. Dissolution experiments were carried out in water for the three cocrystals. While cocrystals with benzoic acid and fumaric acid only show slightly increase on aqueous solubility, the system fluorexitine hydrochloride:succinic acid exhibits an approximately 2-fold increase in aqueous solubility after only 5 minutes (Figure 32).

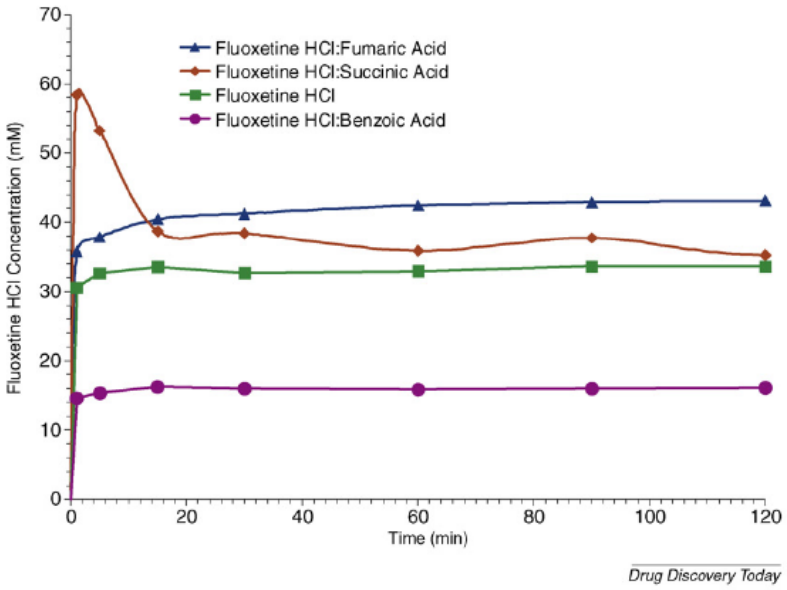

Figure 32: Dissolution profiles for novel cocrystal forms of fluoxetine hydrochloride. Reprinted from [60]

So, simply hydrogen bonding hydrochloride salt of an API with similar coformers can generate different dissolution profiles [60].

4.3.2. Pharmaceutical cocrystals of sildenafil (Viagra®)

Sildenafil is a drug used in the treatment of pulmonary arterial hypertension, congestive heart failure, atherosclerosis, conditions of reduced blood vessel patency and peripheral vascular disease, as well as male erectile dysfunction and female sexual disorders [74]. Sildenafil citrate has been commercially developed and marketed by Pfizer and is available under the trademark Viagra®. Due to its moderate water solubility a study to cocrystallize sildenafil was performed. Sildenafil has been successfully cocrystallized with acetylsalicylic acid, in molar ratio 1:1, by slurry ripening and it has been observed that sildenafil in a pharmaceutical cocrystal form could provide an improved solubility of the API under acidic conditions. The crystal structure of the cocrystal of sildenafil and acetylsalicylic acid has been determined by Single Crystal X-ray Diffraction and the composition of cocrystals was confirmed by X-ray Powder Diffraction and Infrared spectroscopy. Moreover, the Differential Scanning Calorimetry and Thermogravimetric Analyses indicate that the melting point of the cocrystal is approximately 143°C, and it remains thermodynamically stable up to ca. 165°C. In addition, such an improvement of solubility of sildenafil could be particularly advantageous for its orally administrable formulation.

Results

5. Results

This thesis work was focused on the study of solid state properties and polymorphism of different molecules of industrial interest. For this reason, all the information and results obtained from the different studies has to be considered confidential and no one of these is reported in this manuscript. A general description of the performed studies and some not confidential results will be here reported.

5.1. Experimental

5.1.1. Instruments

Here are reported the instruments and the analytical methods used during this thesis work to investigate all the samples obtained from the various crystallization screenings.

X-Ray Powder Diffraction (XRPD)

The crystalline state of samples was investigated by X-ray powder diffraction (D8 Advance, Bruker) equipped with Cu radiation. Samples were placed on zero background sample holder. The measurements were performed in reflection with 2Theta ranging from 3 to 45°.

Single Crystal X-Ray Diffraction (SC XRD)

The structural characterization of obtained single crystals was investigated by the SC XRD D8 Venture®, Bruker equipped with Cu and Mo microfocus source. The measurements were generally performed at 100° K with different methods designed based on unit cell dimension and crystal quality.

Thermogravimetric Analysis (TGA)

TGA analysis was performed using a TA Instruments thermogravimetric analyzer Discovery equipped with a computer analyzing system (TRIOS). Each sample (5-10 mg) was placed in an aluminum sample pan, inserted into the TGA furnace, and accurately weighed. The furnace was first equilibrated at 5 °C, and then heated under nitrogen (flow

rate 30 mL/min) at a rate of 10°C/min, up to a final temperature of 300°C. Nickel was used as the calibration standard.

Differential Scanning Calorimetry (DSC)

DSC analysis was performed using a TA Instruments differential scanning calorimeter Discovery equipped with a computer analyzing system (TRIOS).

About 1-5 mg of each sample were placed into a Tzero Aluminum hermetic DSC pan. The pan was covered with a lid, crimped and pierced in the top lid to prevent pressure build up during solvent or water losses. The sample cell was equilibrated at 0°C and heated under a nitrogen purge (50 mL/min). All samples were given similar thermal histories by linearly heating to 200°C at a heating rate of 10°C/min. Indium metal was used as the calibration standard.

Dynamic Vapour Sorption (DVS)

Moisture sorption/desorption data were collected on a TA Instruments Vapor Sorption Analyzer Q5000SA.

First step: sorption data was collected in the range 40% to 90% relative humidity.

Second Step: desorption, sorption and desorption data were collected over a range of 0% to 90% relative humidity (RH) at 10% RH intervals under a nitrogen purge. Samples were not dried prior to analysis. Equilibrium criteria used for analysis were less than 0.100% weight change in 20 minutes, with a maximum equilibration time of 1 hour if the weight criterion was not met. Data were not corrected for the initial moisture content of the samples. NaBr was used as humidity verification.

Optical microscopy with Polarized light (PLM)

Optical images were acquired using an Axio Imager.M2m, Zeiss polarising microscope, equipped with an Axiocam camera and an image capture software (Axio Vision). The images were recorded using the 10, 20 and 50X objectives. Sample was prepared by deposition of some mg on a microscope slide.

Karl Fischer (KF)

Water content was measured on a 907 Titrando Metrohm, 803 Ti Stand, by the use of a volumetric titration cell. About 15-20 mg of sample were accurately weighed for the water

content determination. Water was used to verify the water content on the replicates with an acceptance RSD criteria < 3%. The instrument control and data analysis software was Tiamo.

Crystallization systems

Polar Bear Plus® by Cambridge Reactor Design and Crystal16® by Technobis were used for slurry at room temperature and temperature cycling experiments.

5.1.2. Crystallization experiments

The following crystallization experiments were performed during this thesis work.

Slow evaporation

Weighted samples (ca. 15 mg each) were put in 4mL vials and they were evaporated at ambient conditions. The solvent was allowed to evaporate by piercing the septum through a needle or by removing the lid. Any solids obtained were analyzed at least with XRPD and optical microscopy.

Slurry ripening at room temperature

Weighted samples (ca. 15 mg each) were put in 2mL crimped vial and treated with different solvent systems. Samples were slurried at room temperature in a Polar Bear Plus system. After different days samples were evaporated with a GreenHouse system and any solids produced were analyzed at least with XRPD and optical microscopy.

Maturation with temperature cycling

Weighted samples (ca. 15 mg each) were put in 2mL crimped vial and treated with different solvent systems. Samples were subjected to heat-cool cycles, generally between 5 and 50ºC, 4h at each step, in a Polar Bear Plus® system. After different days samples were evaporated with a GreenHouse system and solid powders obtained were analyzed at least with XRPD and optical microscopy.

Maturation with temperature cycling using Crystal16® system

Weighted samples (ca. 5 mg each) were put in 2mL crimped transparent vial and treated with different solvent systems to obtain a concentration of 5 mg/mL for each sample. A Crystal16® automated multi-reactor was used and the following method was applied:

- Heat ramp at 5°C/min to a high temperature (depending on the boiling point of solvent)
- Hold at the maximum temperature for 5 min
- Cool ramp at 0.5°C/min to -15°C
- Hold at -15°C for 60 min
- Magnetic stirring at 1000 rpm

Samples were evaporated with a GreenHouse system and solid powders obtained were analyzed at least with XRPD and optical microscopy.

Cooling crystallization

Crystallization can be obtained by lowering the temperature of a clear solution. The solubility of most materials decreases with decreasing temperature, so cooling can be used to generate supersaturation. Weighed samples (ca. 15 mg each) were put in 4mL vial and treated with different solvent systems using a Polar Bear Plus system. Samples were solubilized with a slow and not controlled ramp at high temperature (depending on the boiling point of the solvent); if solubilization didn't occur, an aliquot of solvent was added. After solubilization, samples were subjected to a low cooling ramp (1°C/min to 5°C) and then stored to 4°C for different days. Samples were then evaporated with a GreenHouse system and solid powders obtained were analyzed at least with XRPD and optical microscopy.

Vapor diffusion

Samples (ca. 100 mg) were weighed in 4mL vials, dissolved in different solvent systems (2-3 mL) and dispensed in the inner vials. Different selected volatile anti-solvent systems (5-6 ml) were placed in the outer larger vials. The large vials were closed and the systems were kept at RT. Samples from vapor diffusion experiments were isolated after different days: those experiments still showing solutions were subjected to evaporation by removing the vial lids. Solid powders obtained were analyzed at least with XRPD and optical microscopy.

Co crystallization by milling with Planetary ball mill Pulverisette 6

Samples (ca. 50 mg) were weighed in 4mL vials, zirconia silica spheres were added in order to obtain a ratio 1:5 w/w compound : spheres. A milling process was performed with Planetary ball mill Pulverisette 6, by Fritsch, using those parameters: 300 rpm speed, 1 hour (30 minutes in reverse mode) time. Solid powders obtained were analyzed at least with XRPD and DSC.

5.2. Case studies

5.2.1. Case 1

Different crystallization studies were performed on a molecule which showed low solubility, a potential issue for the *in-vivo* distribution and for the potential of accumulation of API in the lung. Deep investigations were performed on the already available crystalline forms of the free base, named A and B, both equally low soluble. Regarding form B, an hot stage microscopy trial was performed applying a temperature cycle of 25°C-150°C-25°C at 2°C/min (Figure 33); the experiment showed solid phase transition upon heating, confirmed by Variable Temperature XRPD and DSC (data not reported). Unfortunately this potential new form has never been isolated in order to be further characterised.

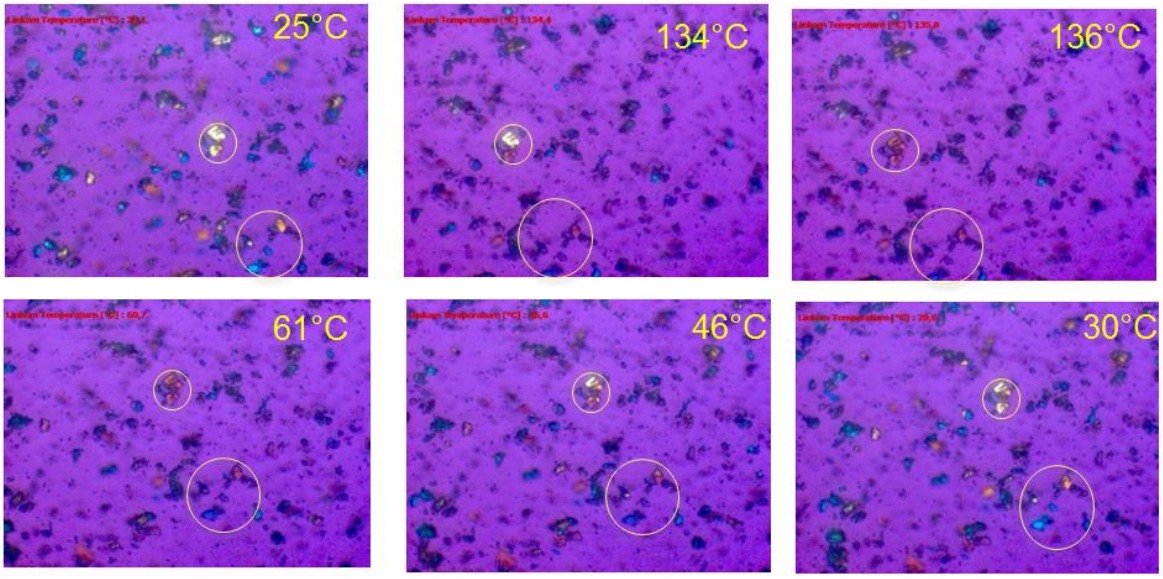

Figure 33: Hot stage microscopy temperature cycle shows a solid phase transition of form B.

In order to obtain a crystalline form with increased solubility the following studies were performed.

- One salt screening using 14 counter ions and 9 solvent systems with the following crystallization techniques

    - Temperature cycling
    - Slow cooling crystallization
    - Temperature cycling followed by anti-solvent addition and slurry ripening at room temperature (RT)
    - Slow evaporation
- One polymorph screening on two amorphous salts using 10 solvent systems with the following crystallization techniques
    - Vapor diffusion
    - Slurry ripening at RT
- One cocrystal screening using 2 different co-formers by wet and dry manual grinding (data not reported)

A more detailed description of the experiments and visual results are reported in the tables below. All the obtained solid samples have been analyzed by XRPD, DSC and optical microscopy, but, due to confidentiality reasons, no data are reported in this thesis.

Technique	Compound	Solvent	Counterion	Temp cycling -15°C-25°C	Antisolvent addition + cooling at -15°C 24h	Slow evaporation at RT
Temperature cycling	Form B	MeOH	H_2SO_4 2M	Solution	0.5 mL MTBE	Sticky solid + white powder
		MeOH	HCl 2M	Solution	0.5 mL MTBE	Sticky solid + white powder
		EtOH	Oxalic acid 5M	Solution	NA	White powder
		EtOH	HCl 5M	Solution	NA	White powder
		MeCN	Oxalic acid 5M	Suspension	NA	White powder
		MeCN	HCl 5M	Suspension	NA	White powder
		EtOAc	Oxalic acid 5M	Suspension	NA	White powder
		EtOAc	HCl 5M	Suspension	NA	White powder
		THF	Oxalic acid 5M	Suspension	NA	White powder
		THF	HCl 5M	Suspension	NA	White powder
	Amorphous	MeOH	H_2SO_4 2M	Solution	0.5 mL MTBE	White powder
		MeOH	HCl 2M	Solution	0.5 mL MTBE	Sticky solid + white powder

Table 4: Salt screening - Temperature cycling experiment results

Technique	Compound	Solvent	Counterion	Cooling -15°C 24h	Other steps	Slow evaporation at RT
Slow cooling	Form B	MeOH	H_2SO_4 2M	Solution	+H_2SO_4 2M	White crystal
		MeOH	HCl 2M	Solution	+HCl 2M	White powder
	Amorphous	MeOH	H_2SO_4 2M	Solution	+H_2SO_4 2M	White crystal
		MeOH	HCl 2M	Solution	+HCl 2M	White pink powder

Table 5: Salt screening - Slow cooling experiment results

Technique	Compound	Solvent	Counterion	Heat cool +60ºC -15ºC	Slow evaporation at RT	Antisolvent addition+slurry RT 24h	Greenhouse evaporation
Temperature cycling + antisolvent addition + slurry at RT	Form B	EtOH	H_2SO_4 18.3M	Suspension at 0ºC	Oil	0.2 mL EtOAc	Oil
		EtOH	HCl 12.07M	Solution	Oil	0.2 mL EtOAc	White powder
		THF	H_2SO_4 18.3M	Suspension at RT	Oil	0.2 mL EtOAc	Oil
		THF	HCl 12.07M	Suspension at RT	Oil	0.2 mL EtOAc	White powder
		EtOH	Maleic acid 1M	Suspension	Pink oil	—	—
		EtOH	Fumaric acid 1M	Suspension	Pink powder	—	—
		THF	Maleic acid 1M	Suspension	Pink oil	—	—
		THF	Fumaric acid 1M	Suspension	Pink oil	—	—

Table 6: Salt screening - Temperature cycling + antisolvent addition + slurry at RT experiment results

Technique	Compound	Solvent	Counterion	Slow evaporation at RT
Slow evaporation	Form B	MeOH	H_2SO_4 2M	Few white crystals
			HCl 2M	Trasparent crystals
			Fumaric acid 1eq	Light pink powder
			Maleic acid 1eq	Light grey powder
	Amorphous	THF	H_2SO_4 2M	Trasparent oil
			HCl 2M	Trasparent oil
			Fumaric acid 1eq	Pink oil
			Maleic acid 1eq	Orange oil

Table 7: Salt screening - Slow evaporation experiment results

Compound	Antisolvents	Solvents	
		EtOH	H_2O
Salt 1	Heptane	Yellow sticky solid	NA
	EtOAc	Brown oil	NA
	MTBE	Yellow sticky solid	NA
	Diisopropyl ether	Yellow sticky solid	NA
	THF	Yellow oil	Oil
	MeCN	Yellow oil	Oil
	Dioxane	Brown oil	Oil

Compound	Antisolvents	Solvents		
		THF	Acetone	EtOH
Salt 2	H_2O	Red oil	Violet oil	Pink sticky solid
	Diisopropyl ether	Withish solid	Pink crystals in solution	Pink sticky solid
	EtOAc	Pink oil	Pink sticky solid	Pink oil
	Heptane	Pink crystals in solution	Violet oil	Pink oil

Table 8 (a and b): Polymorph screening - Vapor diffusion experiment results

Compound	Solvent	Slurry at RT	Greenhouse evaporation/antisolvent addition	
Salt 1	Ethyl formate	beige suspension	Evaporation	NA
	DIPE	beige suspension	Evaporation	NA
	EtOAc	beige suspension	Evaporation	NA
	EtOH	solution	400µL heptane +4ºC	White suspension
	Nitromethane	yellow oil-solution	400µL heptane +4ºC	Solution / sticky solid
	MeCN + 5% H2O	solution	400µL heptane +4ºC	Solution / sticky solid

Compound	Solvent	Slurry at RT	Greenhouse evaporation	
Salt 2	Ethyl formate	beige suspension	Evaporation	Pink sticky solid
	DIPE	beige suspension	Evaporation	White powder
	EtOAc	beige suspension	Evaporation	Pink powder
	Nitromethane	yellow oil-solution	Evaporation	Yellow solid

Table 9 (a and b): Polymorph screening - Slurry experiment results

At the end of these studies no crystalline hits with increased solubility were found.

5.2.2. Case 2

Crystal growth techniques were employed to obtain suitable single crystal, for structural determination by Single Crystal X-ray Diffraction (SC XRD), of a very complex molecule of industrial interest. The following experiments were set up; at least 40 solvent systems were used for each study:

- Solvent evaporation
    - at RT (w/o anti-solvent addition)
    - at 5°C (w/o anti-solvent addition)
    - at 50°C (w/o anti-solvent addition)
- Maturation between 5-50°C (up to 25 days)
- In situ salification
    - Addition of stoichiometric counter ions to free base
    - Maturation between 5-50°C
- Vapor diffusion with few solvents (due to low solubility of the molecule)
- Cooling crystallization
    - Solubilisation at 50°C, slowly cooled to 5°C and then stored at 5°C up to 1 month

In addition to these crystallization techniques, gel crystallization experiments were used as a tool to deep investigate this very complex molecule. The precipitation approach was used (Figure 18) and the gel type selected was PEO. Different gelifications trials were performed with the following solvent systems in order to identify a solvent able to solubilize both PEO and the molecule:

- Dichlorometane
- Acetonitrile
- Water
- Nitromethane
- Chloroform
- Methanol/Water : 50/50
- Tetrahydrofuran /Water : 50/50
- Ethanol/Water : 50/50

In Figure 34 an optical microscopy image of some crystals obtained by gel experiments is showed; unfortunately the good crystals obtained resulted to be the counterion alone.

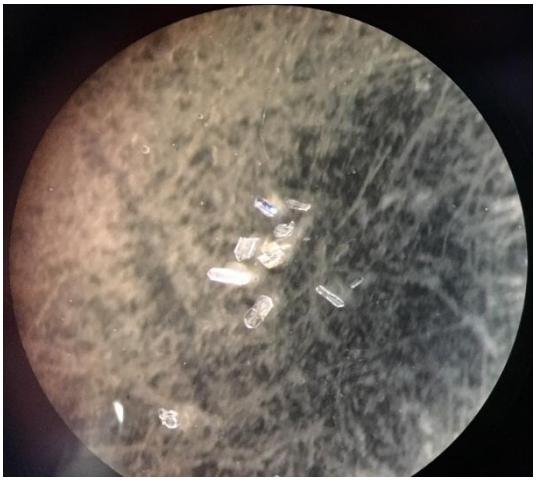

Figure 34: Crystals obtained by gel experiments

At the end of the mentioned studies, at least 50 solid samples obtained were analysed at Polarized Light Microscope in order to identify a suitable single crystal for the analysis. Finally, a single crystal of 0.40 x 0.05 x 0.04 mm of dimensions was found and it has been measured with the D8 Venture® SC XRD, Figure 35. This is an example of the valuable performances offered by this equipment, which allowed to elucidate the molecular structure of a very complex compound using an extremely tiny crystal.

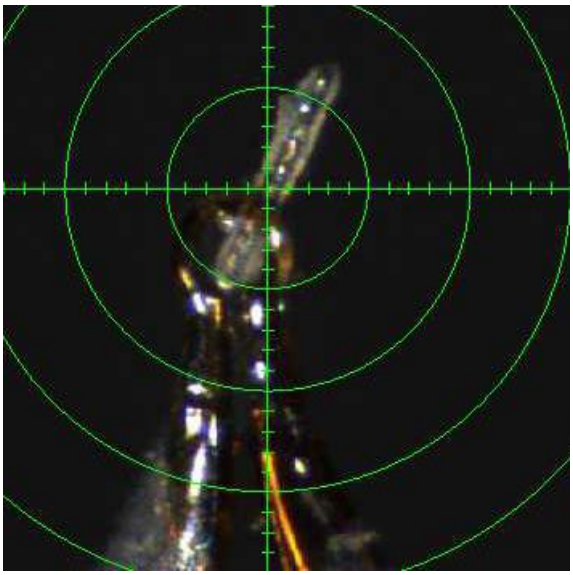

Figure 35: Image of the single crystal analysed

The structural information obtained with this analysis were used to support the characterization of the molecule and better understand its developability. Results obtained by the Single Crystal analysis cannot be shown due to confidential reasons.

5.2.3. Case 3

A semi-automated approach for generation and analysis of co crystals was explored, employing ball milling in a planetary mill with a sample holder with 8 positions and DSC. The work was performed on two active ingredients of academic interest (A and B) combined with two different co formers (C and D), with the aim to translate the results for an industrial use in order to enhance the use of co crystal screening during polymorphism study of NCEs. In this case study, a complete characterization via SC XRD and DSC of the cocrystals AC, AD, BC and BD was already available from previous work. The SC XRD images of co crystals obtained by grinding or by direct mixing are here reported (Figure 36-40) [75].

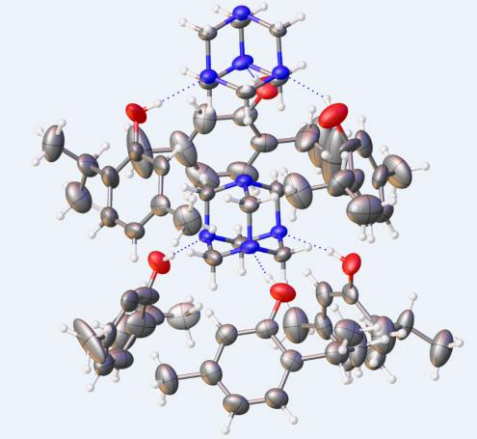

Figure 36: Supramolecular interactions in the crystal structure of co crystal AC, showing also the thermal displacement ellipsoids at the 50% probability level. Hydrogen bonds are represented as dotted lines

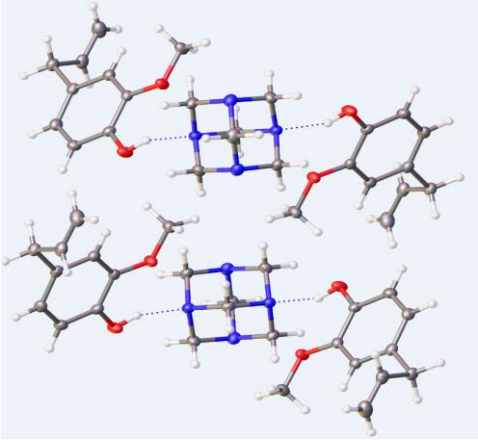

Figure 37: Supramolecular interactions in the crystal structure of co crystal AD, showing also the thermal displacement ellipsoids at the 50% probability level. Hydrogen bonds are represented as dotted lines

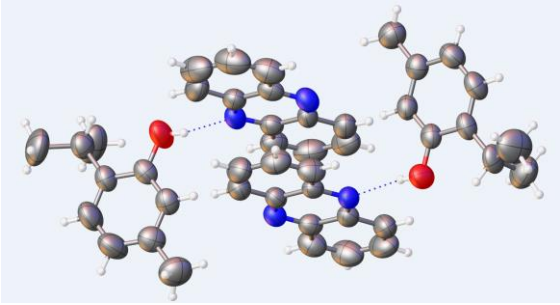

Figure 38: Supramolecular interactions in the crystal structure of co crystal BC, showing also the thermal displacement ellipsoids at the 50% probability level. Hydrogen bonds are represented as dotted lines

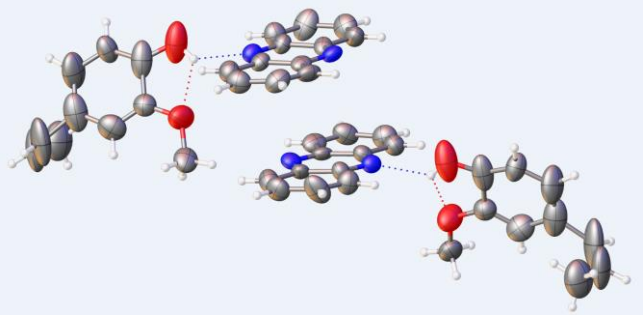

Figure 39: Supramolecular interactions in the crystal structure of co crystal BD, showing also the thermal displacement ellipsoids at the 50% probability level. Hydrogen bonds are represented as dotted lines

The focus of the experimental work was to investigate standard screening protocols which could be scaled up to industrial use. To set up the approach, twelve experiments were performed using different co formers and different stoichiometry.

The steps of the screening are reported below:

1. DSC ramp on single components A, B, C and D to confirm melting points
2. Grinding of each of the ingredients together with the selected coformers using in the planetary ball mill with an 8 positions sample holder, in different conditions (time, rpm, material/dimension of spheres)
3. DSC ramp on obtained powders to verify co crystals formation
4. XRPD

Each sample was added with a predetermined number of spheres and treated with a selected method in a planetary ball mill in order to obtain a solid co crystal. Different processing conditions were evaluated, testing various combinations of time, rpm, material and dimension of spheres.

Results from experiments using molecules of academic interest are here reported. The experiments have been performed using the planetary mill with the following conditions:

- Speed: 300 rpm
- Time: 1 h (30 min reverse)
- Spheres: zirconia silica of two dimension, 1 mm and 2.3 mm

In addition, in some experiments a ratio (weight/weight) of co formers/spheres:1/1 was used, while in other experiments no spheres were added to co formers.

Sample name	Co former 1	Co former 2	Co former ratio	Ratio (w/w) co formers/spheres
Experiment 1	Molecule A	Molecule C	1:3	NA
Experiment 2				1:1
Experiment 3		Molecule D	1:2	NA
Experiment 4				1:1
Experiment 5	Molecule B	Molecule C	1:3	NA
Experiment 6				1:1
Experiment 7		Molecule D	1:2	NA
Experiment 8				1:1
Experiment 9		Molecule C	1:1	NA
Experiment 10				1:1
Experiment 11		Molecule D	1:1	NA
Experiment 12				1:1

Table 10: Co crystallization experiments

Solids obtained from those experiments were analysed by DSC and XRPD. Results are reported below.

Figure 40 and Figure 41 show thermogram and diffractogram resulting from Experiment 1, respectively. A temperature cycle ramp was performed in DSC:

- Ramp 5°C/min from RT to -60°C
- Ramp 5°C/min from -60°C to +100°C
- Ramp 5°C/min from +100°C to -60°C
- Ramp 5°C/min from -60°C to +100°C
- Ramp 5°C/min from +100°C to +20°C

DSC analysis on the ground milled powder obtained from Experiment 1 sample shows a clear endothermic peak at 48.31°C not associated with the melting of the co crystal whose melting point is 39°C. XRPD pattern shows co formers residual presence.

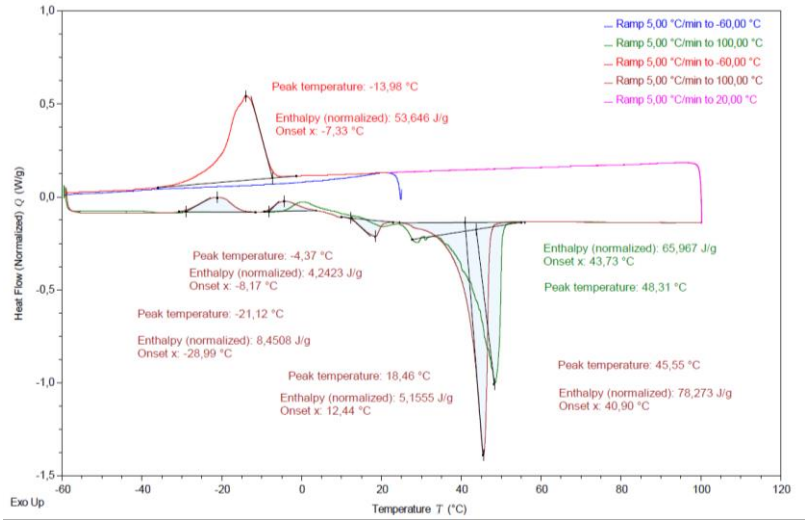

Figure 40: DSC thermogram resulting from Experiment 1

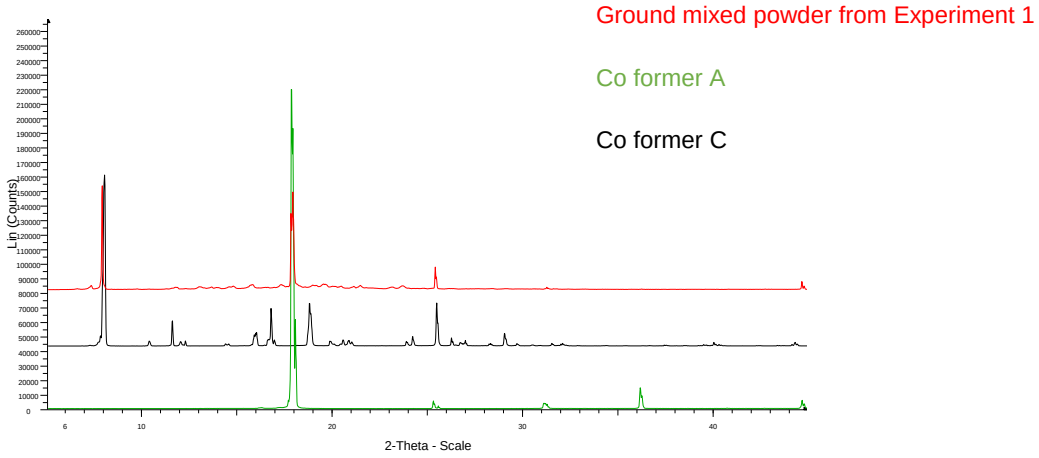

Figure 41: XRPD diffractogram resulting from Experiment 1

Same results were obtained with Experiment 2. The DSC thermogram (Figure 42) shows a clear endothermic peak at 49.98°C not associated with the melting of the co crystal. XRPD pattern (Figure 43) shows co formers residual presence.

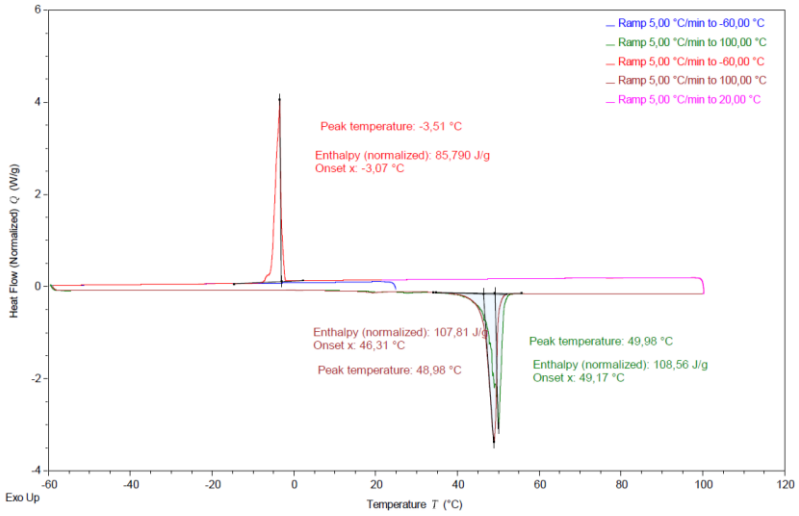

Figure 42: DSC thermogram resulting from Experiment 2

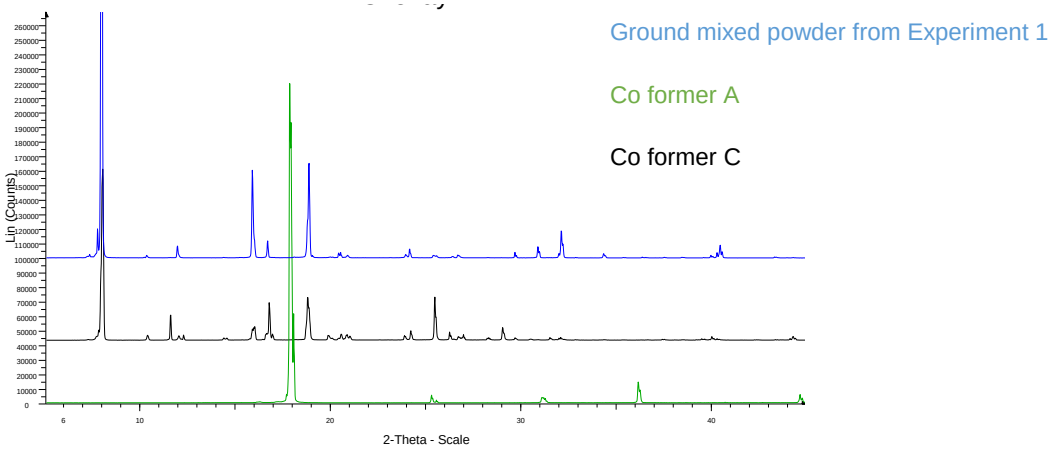

Figure 43: XRPD diffractogram resulting from Experiment 2

DSC analysis on the ground milled powder obtained from Experiment 3 (Figure 44) sample shows a clear endothermic peak at 85.11°C associated with the melting of the co crystal, as analysis on Experiment 4 (Figure 45) shows a clear endothermic peak at 85.13°C associated with the melting of the co crystal. A slurry was obtained, so no XRPD experiment was performed on those samples.

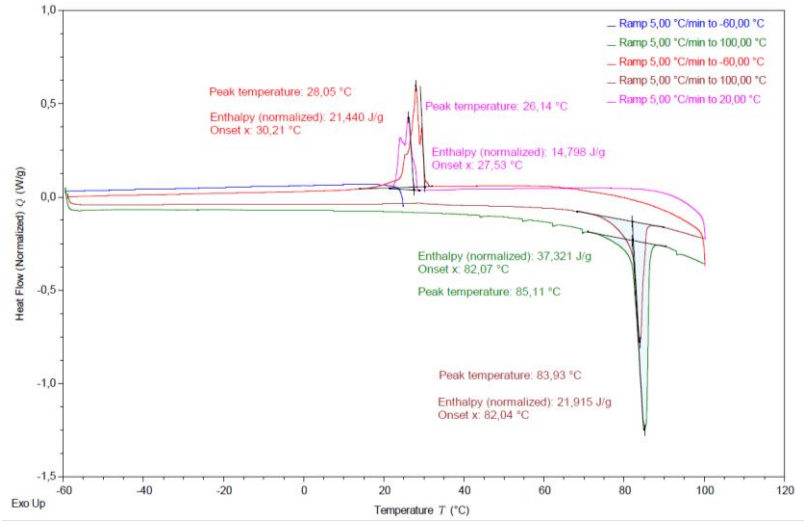

Figure 44: DSC thermogram resulting from Experiment 3

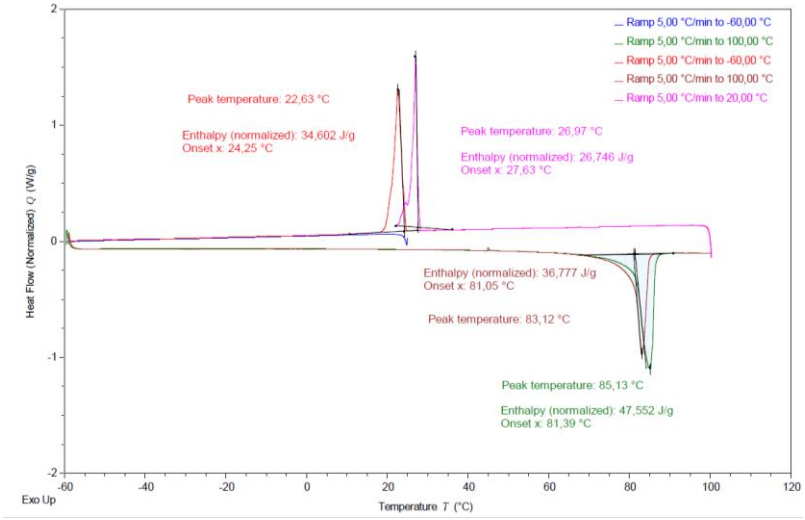

Figure 45: DSC thermogram resulting from Experiment 4

A temperature cycle ramp was performed in DSC on the ground milled powder obtained from Experiment 5 and 6:

- Ramp 5°C/min from RT to -60°C
- Ramp 5°C/min from -60°C to +80°C
- Ramp 5°C/min from +80°C to -60°C
- Ramp 5°C/min from -60°C to +80°C

- Ramp 5°C/min from +80°C to +20°C

Analysis on Experiment 5 (Figure 46) sample shows a clear endothermic peak at 50°C not associated with the melting of the co crystal whose melting point is 87°C. Analysis on Experiment 6 (Figure 48) shows a sharp endothermic peak at 43.69°C, also in this case not associated with the melting of the co crystal. XRPD patterns of both experiments show residual presence of co formers (Figure 47 and Figure 49).

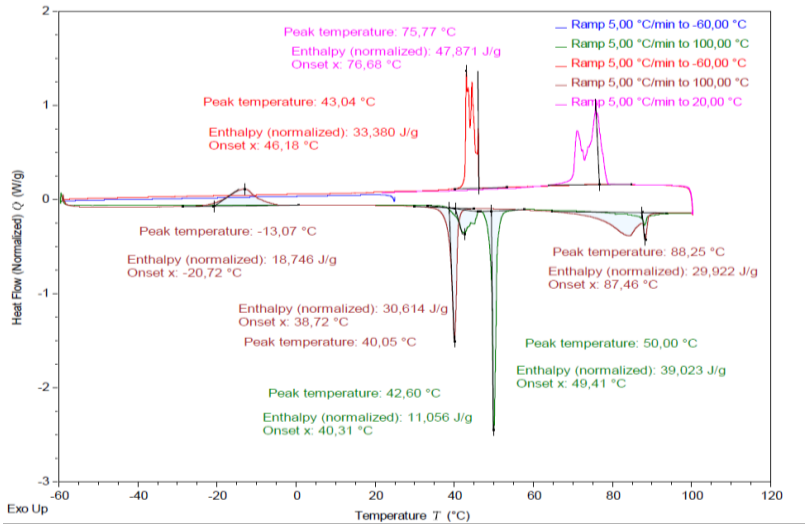

Figure 46: DSC thermogram resulting from Experiment 5

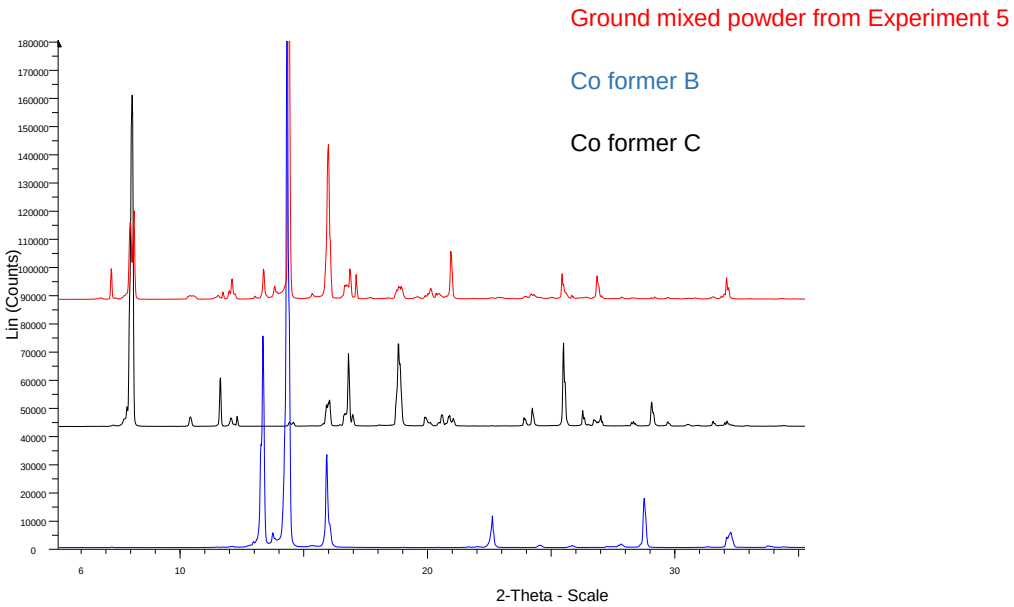

Figure 47: XRPD diffractogram resulting from Experiment 5

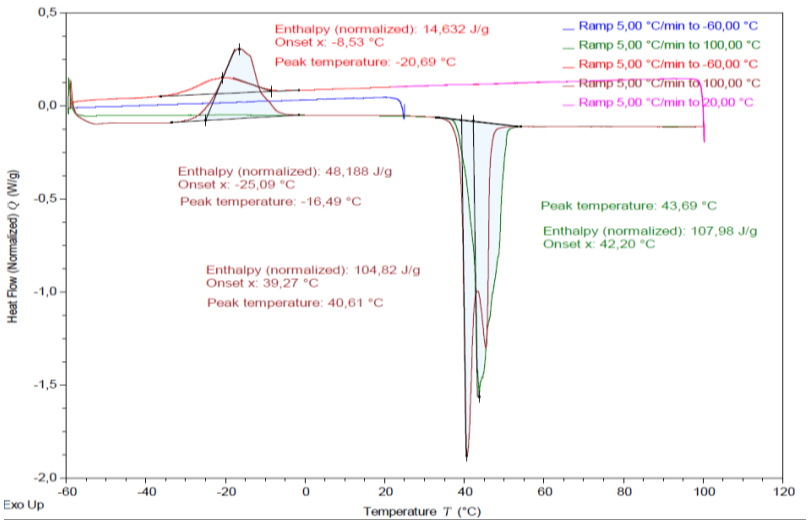

Figure 48: DSC thermogram resulting from Experiment 6

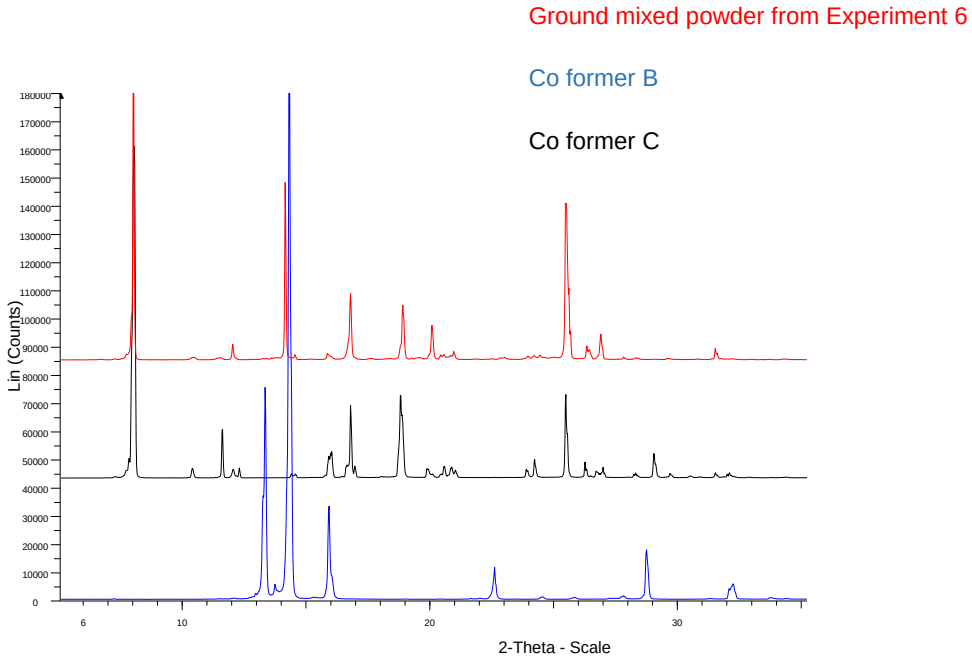

Figure 49: XRPD diffractogram resulting from Experiment 6

DSC analysis on the ground milled powder obtained from Experiment 7 sample (Figure 50) shows a clear endothermic peak at 53.19°C associated with the melting of the co crystal, as analysis on Experiment 8 shows a clear endothermic peak at 53.26°C associated with

the melting of the co crystal (Figure 51). A slurry was obtained, so no XRPD experiment was performed on those samples.

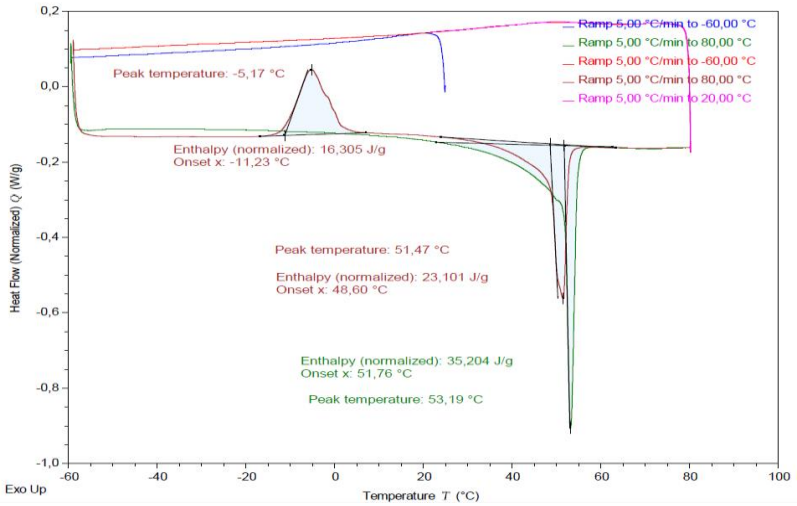

Figure 50: DSC thermogram resulting from Experiment 7

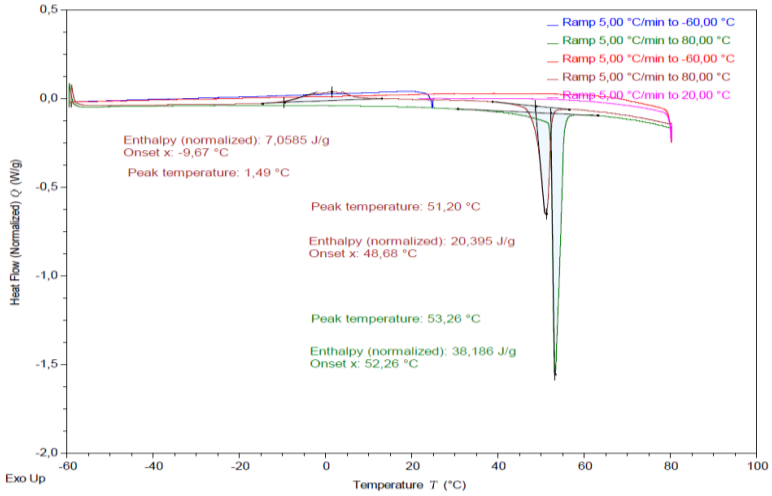

Figure 51: DSC thermogram resulting from Experiment 8

DSC analysis on Experiment 9 sample shows a sharp endothermic peak at 43.41°C not associated with the melting of the co crystal whose melting point is 87°C. Both DSC thermogram and XRPD patterns show residual presence of co formers (Figure 52 and

Figure 53). Same results are obtained with Experiment 10 which shows residual presence of co formers both from DSC and XRPD analyses (Figure 54 and Figure 55).

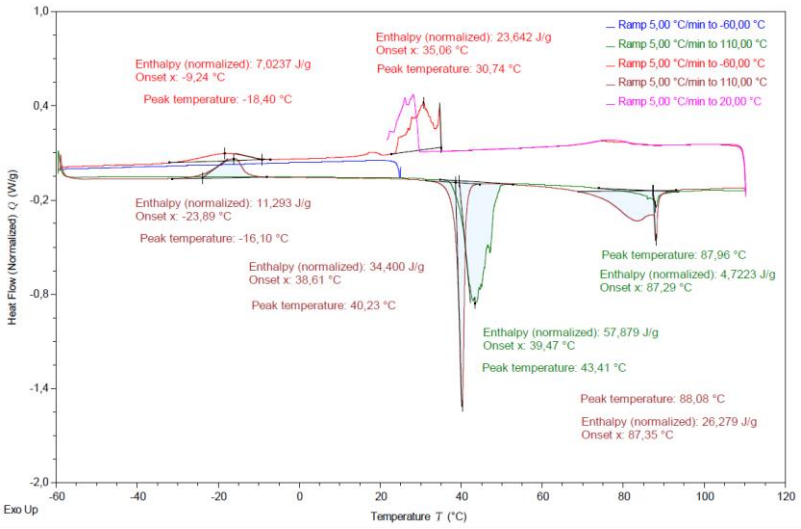

Figure 52: DSC thermogram resulting from Experiment 9

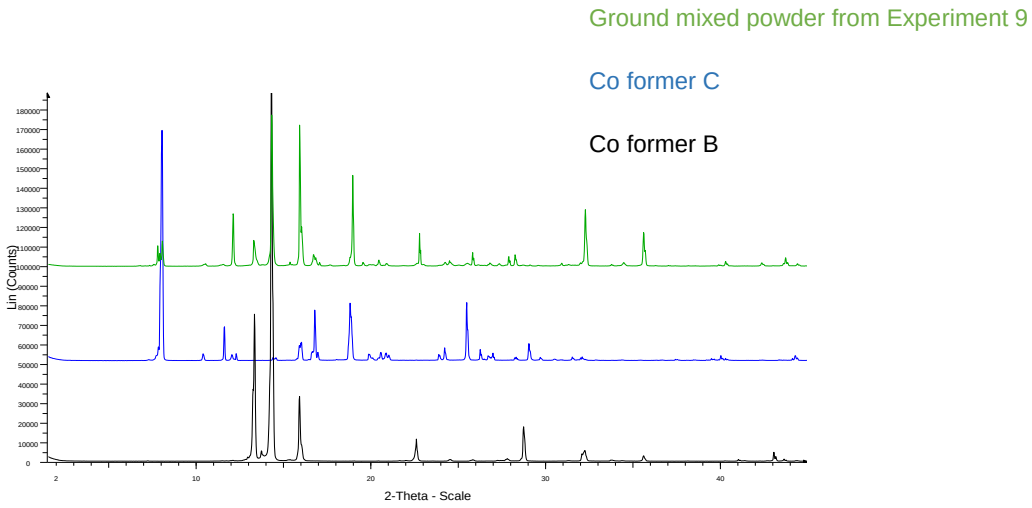

Figure 53: XRPD diffractogram resulting from Experiment 9

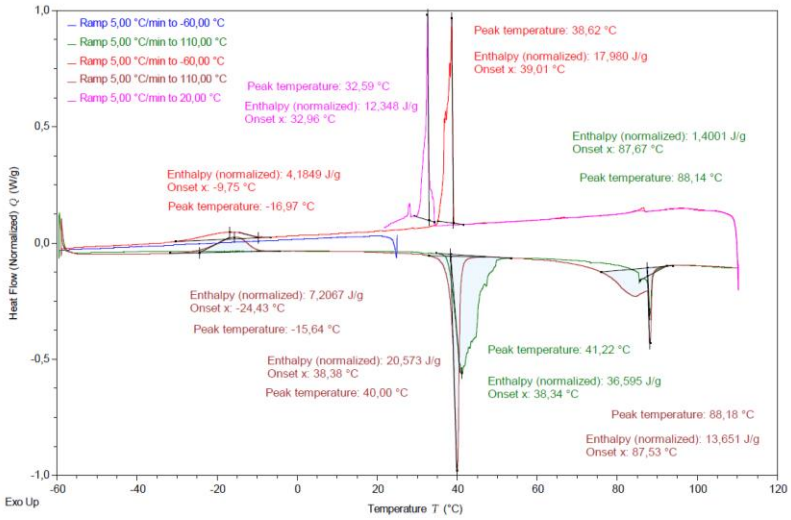

Figure 54: DSC thermogram resulting from Experiment 10

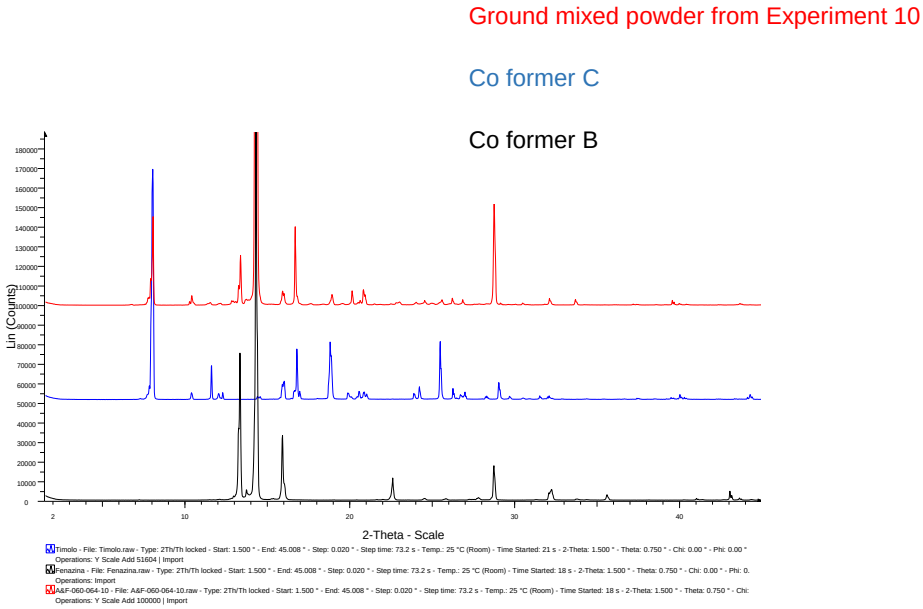

Figure 55: XRPD diffractogram resulting from Experiment 10

DSC analysis on the ground milled powder obtained from Experiment 11 sample (Figure 56) shows a clear endothermic peak at 51.94°C associated with the melting of the co crystal (54°C), as analysis on Experiment 12 shows a clear endothermic peak at 51.89°C

associated with the melting of the co crystal (Figure 57). A slurry was obtained, so no XRPD experiment was performed on those samples.

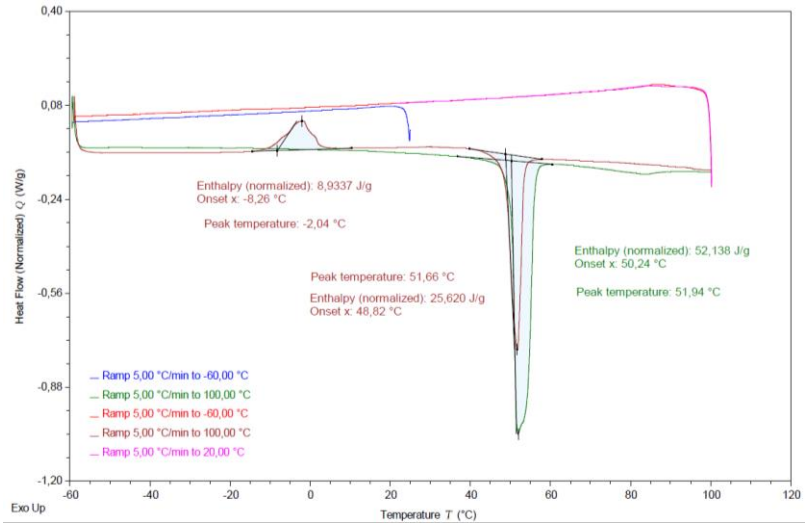

Figure 56: DSC thermogram resulting from Experiment 11

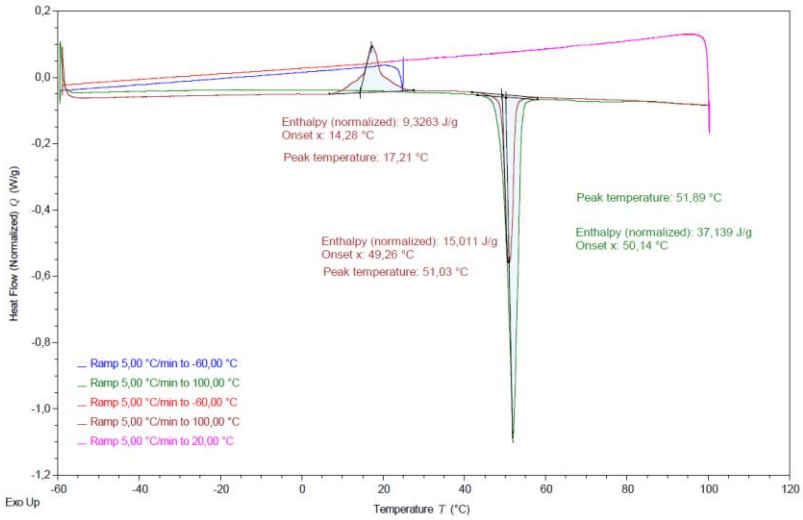

Figure 57: DSC thermogram resulting from Experiment 12

From some of those experiments co crystals were obtained showing the potential value of the application of this approach to a more systematic cocrystal screening with molecules of pharmaceutical interest. On the other hand, different experiments showed residual co

formers presence or no formation of co crystals, probably due to different reasons such as a wrong co formers ratio or an automated grinding program to set up with other parameters. The mechanochemical synthesis of cocrystals is surely a remarkably advantageous procedure which does not involve solvent usage and disposal, and hence it is very important to explore and better understand the processes underlying mechanochemical synthesis. On the other hand, the application of this approach to our systems evidenced that the processes involved in the grinding seem to be more complexes that those of co crystallization in solution, probably involving the formation of transient polymorphic forms, or the evolution of different stoichiometries during a process, as evidenced by the complex shapes of the thermal profiles in most of the cases. This work has shown that a careful study and a deep understanding of the solid state processes involved during grinding is needed before implementing such procedures on a broad scale.

Conclusions

6. Conclusions

This thesis work aimed to give an overview on the role and importance of polymorphism for the pharmaceutical field and on the application of crystallography to help in the evaluation of properties of crystalline solid forms. As mentioned, this is important for all kind of solid dosage drugs because of properties of crystalline solids that allow to have more stable, reproducible and easily handleable Active Pharmaceutical Ingredients (APIs).

However, as discussed in Chapter 1, for the drugs developed for the inhalation administration it is extremely important to start with appropriate crystallization studies in a very early phase of drug development in order to identify criticalities and to select, as soon as possible, a suitable solid form for the future development. Therefore, developing crystallization screening protocols has revealed to be a very useful tool to identify and characterize a large number of New Chemical Entities (NCEs).

During this thesis work, these concepts were practically applied during the laboratory work, mixing approaches and techniques well known and established in the pharmaceutical industry with some others peculiar to the academic world. For example, the gel crystallization was approached in order to have an additional chance during a polymorph screening to obtain and to grow crystals. Moreover, a new approach to screen cocrystals of NCEs was developed with the use of a multi position Planetary Mill and the subsequently analyses with the Differential Scanning Calorimetry (DSC).

Finally, during this thesis work Single Crystal X-ray Diffraction (SC XRD) was used as much as possible in order to add the fundamental structural information given by this technique to the others obtained by other analytical techniques, such as XRPD, DSC, Thermogravimetric analysis (TGA), Infrared and Raman spectroscopy, Nuclear Magnetic Resonance (SSNMR). As mentioned in the previous chapters, thanks to the use of a new generation instrument, such as the D8 Venture® of Bruker, the number of analyses has been increased. In fact, as described in Chapter 2, the D8 Venture® has some technological improvements which allow better and faster analyses. For example, thanks to the presence of the detector Photon 100, with a large active area of 100 cm^2, there is the opportunity to perform fast acquisition with an high data quality. Besides, the presence

of the microfocus source allows the measurement also single crystal of small dimensions (<50μm) and poor quality, thanks to its beam brighter than conventional X-Ray sources. A particular advantage is strongly linked to the availability of the Cu microsource which allows an easily determination of absolute stereochemistry of chemical entities which contain first row elements only. For those reasons, the number of SC XRD analyses performed on molecules of interest has increased in the recent years. The structural characterization of more than 15 New Chemical Entities has been determined allowing to gather many useful information for aiding the evaluation of their developability as inhalation drugs. Moreover, thanks to the reduced time of analysis, the SC XRD technique has been used also for the identification of reaction intermediates when the others traditional techniques couldn't give an unequivocally answer. For example, two reaction intermediates have been analyzed to confirm the exact position of halogenation reactions.

The work has proved the benefits of strong cooperation between academy and industry: cutting edge academic knowledge on advanced crystallographic concepts and techniques has been employed to challenge specific problems of industrial interest, providing useful results on one hand, but also, and more importantly, providing to the industrial partner a high level scientific training on the most modern approaches in the field of crystal engineering, and to the academic partner a direct contact with a large variety of problems related to the application of model concepts to real cases.

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