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Polymeric stabilizers maintaining the saturation solubility of itraconazole nanocrystals after dissolution process

Diploma thesis

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Polymérne stabilizátory udržujúce nasýtenosť roztoku po rozpustení nanokryštálov itrakonazolu

Diplomová práca

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Statement of originality

I declare that the content of this master thesis is my own work. All the sources that were used to create this thesis are incorporated in quotations and subsequently listed in bibliography. This thesis was not submitted for any other degree or other purposes.

In Hradec Králové signature of the author

Abstract

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The increase of bioavailability of poorly water soluble drugs is still an issue. One of the techniques improving aqueous drug substance solubility, and consequently enhancing bioavailability, is formation of nanoparticles. However, the bioavailability is determined by the concentration of the dissolved drug achieved at the time of absorption. This fact emphasizes the importance of the maintenance of the high solubility until the absorption area is reached. Sufficiently stabilised nanocrystalline drugs offer a solution to this problem. In this thesis, the solid nanoparticle formations of an antifungal agent itraconazole (ITZ) are presented. Wet milling was employed to create the nanosuspension stabilised by binary mixture of stabilisers or by a single stabiliser. An aggregation inhibitor Poloxamer 407 (F127) in the combination with a polymeric precipitation inhibitor hydroxypropyl methylcellulose (HPMC) or polyvinyl pyrrolidone (PVP) at different ratios, or a single precipitation inhibitor, were utilised. The nanoscale was determined by dynamic light scattering (DLS) measurements and the crystalline state was confirmed by differential scanning calorimetry (DSC). The solubility tests showed the importance of utilised stabilisers over particle size within nanoscale. The highest solubility levels and the most successful maintenance of high solubility values were obtained in samples containing a single polymeric precipitation inhibitor, followed by binary mixtures with F127 exceeding the amount of HPMC/PVP. The order can be concluded: HPMC>PVP>F127+HPMC>F127+PVP. The physical state (predissolved/solid) of the precipitation inhibitor influences the solubility level. Hygroscopic properties of PVP enhance its affinity to water and thereby increases solubility, the addition of solid

excipient is more beneficial. Postmilling addition of the precipitation inhibitor impacts on the concentration of dissolved drug positively.

Keywords: itraconazole, nanosizing, supersaturated state, polymeric precipitation inhibitors

Abstrakt

Názov diplomovej práce: Polymérne stabilizátory udržujúce nasýtenosť roztoku po rozpustení nanokryštálov itrakonazolu

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Snaha o zvýšenie biodostupnosti vo vode veľmi ťažko rozpustných liečiv je stále otázkou. Jednou z techník zlepšujúcich rozpustnosť liečiv vo vode, a teda následne podporujúcich biodostupnosť, je vytvorenie nanočastíc. Avšak biodostupnosť je určovaná koncentráciou rozpusteného liečiva v čase absorpcie. Táto skutočnosť zdôrazňuje dôležitosť udržania vysokej koncentrácie rozpusteného liečiva až po miesto absorpcie. Dostatočne stabilizované nanokryštalické liečivá ponúkajú riešenie tohto problému. V tejto diplomovej práci je prezentovaná príprava tuhých nanokryštálov anitmykotického agens itrakonazolu (ITZ). K príprave nanosuspenzie bolo použité mokré mletie, nanosuspenzia bola stabilizovaná binárnou zmesou stabilizátorov alebo jediným stabilizátorom. Bol použitý inhibítor agregácie Poloxamer 407 (F127) v kombinácii s polymérnym inhibítorom precipitácie/zrážania hydroxypropylmetylcelulóza (HPMC) alebo polyvinylpyrolidon (PVP) v rôznych pomeroch, prípadne samotný inhibítor precipitácie. Nanorozmery boli stanovené pomocou dynamického rozptylu svetla (DLS), kryštalický stav bol potvrdený pomocou diferenciálnej skenovacej kalorimetrie (DSC). Testy rozpustnosti ukázali, že použité stabilizátory zohrávajú dôležitejšiu rolu ako veľkosť nanočastíc. Najvyššiu úroveň rozpustnosti a najvýhodnejšie udržanie rozpustnosti boli dosiahnuté vzorkami obsahujúcimi samotný polymérny inhibítor precipitácie, nasledujú vzorky tvoriace binárnu zmes s F127 prevyšujúcim množstvo HPMC/PVP. Poradie môže byť zhrnuté nasledovne HPMC>PVP>F127+HPMC>F127+PVP. Fyzikálny stav (rozpustený alebo tuhý) inhibítora precipitácie ovplyvňuje úroveň rozpustnosti. Hygroskopické vlastnosti PVP posilňujú jeho afinitu k vode, čo vysvetľuje nárast rozpustnosti,

prídavok tejto pomocnej látky v tuhom skupenstve je výhodnejšie. Pridanie polymérneho inhibítora precipitácie po mletí častíc pozitívne ovplyvňuje koncentráciu rozpusteného liečiva.

Kľúčové slová: itrakonazol, príprava nanočastíc, presýtený stav, polymérne inhibítory precipitácie

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1 Aims

The overall aim of this thesis is to analyse the influence of nanosizing in concurrence with combination of stabilisers on solubility level and on maintenance of the supersaturated state.

This thesis provides further information about maintenance of the supersaturated state of nanocrystalline itraconazole (ITZ). To stabilize both the ITZ nanocrystals and the supersaturated state after dissolution, hydroxypropyl methyl cellulose (HPMC), polyvinylpyrrolidone (PVP) and subsequently their binary mixtures with poloxamer 407 (F127) are utilized. Different compositions of mixtures are investigated in order to evaluate influence of following parameters/process variables on solubility and maintenance of supersaturated state:

1. particle size of nanocrystals,
2. choice of stabiliser/combination of stabilisers and its/their concentration,
3. physical state (solid or dissolved) of stabiliser, and
4. addition stage of stabiliser.

2 Introduction

New technologies influence and facilitate our everyday life. Fast progress can be observed in all aspects of human´s life, including pharmaceutical sciences.

Pharmaceutical chemistry shifted towards new methods that enable synthesis of larger number of compounds. Combinatorial chemistry accelerates drug discovery procedures followed by high throughput screening, allowing fast analyses of biological activity. Nevertheless, the fast development in the pharmacy field, leading to a lot of new potential candidates, does not necessarily lead to increase in launched products administrated orally, the most convenient dosage form. The bioavailability, conditioned by solubility and permeability, is often hindered by chemical and physical properties of the drug. Combinatorial chemistry mostly introduces new molecules of high molecular weight and increased lipophilicity that leads to solubility decrease. The solution to this issue can be found at the technological level. The biological activity of the molecule is preserved and the properties influencing the bioavailability are modified to achieve desired plasma concentration of the administrated drug.

However, not only newly discovered drugs may suffer from disadvantageous solubility profile. It is also the case of some medicaments already utilised in therapy. As an example representing group of products exhibiting poor water solubility, itraconazole can be introduced. Itraconazole is an antifungal drug acting against a broad spectrum of agents. As a weak base, the solubility decreases with reduced gastric acidity. The observed oral bioavailability of the current market formulation Sporanox® Capsules is 55 % of administrated amount (Janssen Pharmaceuticals, 2014). Enhanced bioavailability, 30 – 33 % greater, is achieved with Sporanox® Oral Solution (Janssen Pharmaceuticals, 2003), which takes advantage of formulation employing cyclodextrin (Barone et al., 1998).

Several methods have already been applied to improve water solubility and are indivisible part of current pharmacotherapy. The technology has proceeded from simple saltification of weak acids and bases through co-solvents, complexation (e.g. cyclodextrins) and lipid based drug delivery systems to formulations achieving supersaturated state. Regarding supersaturable formulations, not only the level of supersaturation, but also the ability to maintain such high level of dissolved drug, are important. There are two main techniques dealing with reaching and effective

maintenance of the supersaturated state, amorphisation and nanocrystalline formulation. Amorphous composition of various drugs has already been investigated, such as of felodipine (Konno, Handa, Alonzo, & Taylor, 2008) or of indomethacin (Surwase et al., 2015), DiNunzio and co-workers drawn their attention to amorphous ITZ (DiNunzio, Miller, Yang, McGinity, & Williams, 2008; M. A. Miller et al., 2012). Nanocrystalline formulation of ITZ has been widely studied as well. Previous research has documented the influences of stabilisers inhibiting aggregation on particle size (Liu et al., 2011; Van Eerdenbrugh et al., 2009), and the dissolution of ITZ nanocrystals was investigated (Badawi, El-Nabarawi, El-Setouhy, & Alsammit, 2011; Sarnes et al., 2014).

Nevertheless, the attention has been paid only partly to the methods maintaining the supersaturated state. The reference point can be found in Ueda and co-worker´s paper (Ueda, Higashi, Yamamoto, & Moribe, 2015) suggesting longer persistence of concentration of carbamazepine dispersion partially consisting of nanoparticles. Therefore, nanosizing approach has been selected to overcome this existing gap and attempt to maintain the high concentration of dissolved drug after dissolution of nanocrytalline drug. In this study, the maintenance of high concentration after the fast dissolution of nanocrystals with the aid of polymeric precipitation inhibitors were employed (Warren, Benameur, Porter, & Pouton, 2010) and combined with aggregation inhibitor which enables creation of nanosized particles. Various physical stage (predissolved/solid) and addition stage of precipitation inhibitor were also examined.

3 Theoretical section

3.1 Poorly water soluble drugs

Over past years the number of new chemical entities has increased. The combinatorial chemistry and high throughput screening accelerate production of new compound, and production techniques influence drug properties. Using these techniques, the properties have shifted towards higher molecular weight and increased lipophilicity (Lipinski, 2000). Both of these properties are disadvantageous when considering use of such compound in oral drug delivery.

In consequence, high molecular weight and lipophilicity lead to poor water solubility that results in low plasma concentration of such a drug delivered orally. Therefore, higher doses have to be administrated to achieve sufficient pharmacological effect. Higher amount of exogenous compounds burdens elimination organs, mainly liver and kidney. Also patients are bothered with more frequent administration which may decrease patients´ compliance to therapy.

Therefore, attention has been drawn to development of formulation methods that address the solubility issues. These formulation methods are important mainly for drugs classified as BCS (Biopharmaceutics classification system) class II (poorly soluble/permeable), but also for BCS class IV (poorly soluble/poorly permeable)(Amidon, Lennernäs, Shah, & Crison, 1995).

3.1.1 Methods improving water solubility

The orally administered drug must exhibit water solubility and permeability to a certain extent to achieve sufficient bioavailability. Low oral bioavailability is mostly the result of poor water solubility. Nevertheless, solubility of drug material is also influenced by the dissolution environment. Regarding orally administered drugs, the dissolution conditions are strictly given by human gastrointestinal tract. The temperature, approximately 37 ℃, composition and amount of digestive juices and changing pH, by passing through the gastrointestinal tract, must be taken into account.

Many techniques have been designed, such as use of cyclodextrins and co-solvents, to improve water solubility. Historically, salt formation is one of the first methods utilised to dissolve drugs. Salt formation, or saltification, applies to weak

acids and bases. It introduces a charge into molecule that attracts polar water molecule. The choice of counterion influences solubility as well. For instance, diclofenac potassium exhibit superior solubility to diclofenac sodium (Ahmad et al., 2010; Chuasuwan et al., 2009). Cyclodextrins complex poorly water-soluble molecules in their hydrophobic core whereas their hydrophilic surface interact with polar water molecules. Thus, solubility and dissolution rate increase (Brewster & Loftsson, 2007). In pharmaceutical technology the addition of co-solvents to water is limited due to possible toxicity. The lipid based drug delivery systems use the fact, that drugs with poor aqueous solubility may display greater solubility in lipid medium. Hence, drugs are dissolved in lipid phase of stable emulsions. Different kind of lipid based systems like self microemulsifying drug delivery systems or self nanoemulsifying drug delivery have been discovered (Gurram et al.). Formulations that reach the supersaturated state (Kawakami, 2015) are for example amorphous solid dispersions and nanocrystal formulations. Amorphous state is high energy state and thus the solubility of amorphous form is higher compared to low energy forms, such as crystalline form. Amorphous solid dispersion consists of amorphous active pharmaceutical compound that is stabilised by polymer. This combination creates a water-soluble system (Newman, Nagapudi, & Wenslow, 2015). The faster dissolution of nanocrystals rests mainly on the increased surface area. Nanocrystals are introduced more profoundly in the following chapter.

3.1.2 Itraconazole

Itraconazole (ITZ) is a broad-spectrum antifungal agent belonging to group of triazole derivatives. It is orally administrated drug that acts against a diverse range of fungal infections such as blastomycosis, histoplasmosis, aspergillosis and onychomycosis (Janssen Pharmaceuticals, 2014). ITZ plays a significant role as prophylaxis against opportunistic fungal infections in immunocompromised patients (Böhme et al., 1996; McKinsey et al., 1999). In majority of cases, the dose of 200 mg once or twice a day is indicated (Janssen Pharmaceuticals, 2014).

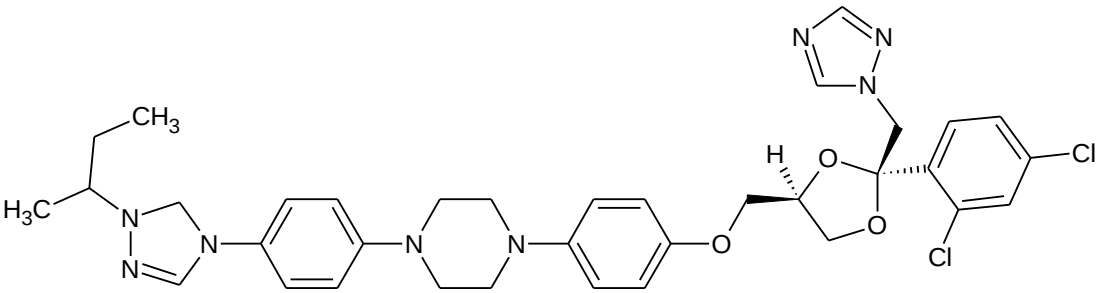


Figure 1. Chemical structure of itraconazole

ITZ (Figure 1) is a lipophilic compound that is practically insoluble in water, very slightly soluble in alcohols, and freely soluble in dichloromethane. Chemical properties of ITZ are summarised in Table 1.

ITZ is a weak base that is protonated at acidic pH 1 of the stomach. Thus, solubility raises up to app. 4 μg/ml. The pH shift to neutral pH in the small intestine, where ITZ is not protonated any more, causes the considerable solubility decrease to app. 1 ng/ml (Peeters, Neeskens, Tollenaere, Van Remoortere, & Brewster, 2002). On the contrary, permeability of this drug is appropriate at a dose of 100 mg. Therefore it is consider as class II in BCS (Sarnes et al., 2014). Good level of permeability indicates that the solubility is the issue that limits bioavailability. Thus, the optimisation of the solubility level may result in improved bioavailability.

Table 1. Properties of ITZ

Property	**Itraconazole**	**Reference**
Log P (n-oct/aq buffer pH 8.1)	5,66	(O'Neil, 2006)
Molecular weight	705 g/mol	(O'Neil, 2006)
Melting point	166,2 °C	(O'Neil, 2006)
Solubility in water	Practically insoluble (more than 10 000 ml of solvent per gram of solute)	(European Pharmacopoeia, 2014a)
Solubility in alcohols	Very slightly soluble (from 1 000 to 10 000 ml per gram of solute)	(European Pharmacopoeia, 2014a)
Solubility in methylene chloride	Freely soluble (from 1 to 10 ml per gram of solute)	(European Pharmacopoeia, 2014a)
pKa	3,7	(Al-Badr & El-Subbagh, 2009)

3.2 Nanocrystals

Drug nanocrystals can be defined as particles with dimensions reduced to nanoscale consisting of solid crystalline drug core and stabiliser layer on its surface. In technical fields, term "nanoparticles" signifies at least one dimension of the system is smaller than 100 nm. But regarding nanocrystalline drugs, their size ranges characteristically from 100 nm to 400 nm, and in pharmaceutical sciences nanocrystals are generally particles below 1000 nm (Peltonen, Hirvonen, & Laaksonen, 2013b).

3.2.1 Nanocrystals and solubility

There are three main items which clarify the advantageous use of nanocrystal in drug delivery (Peltonen et al., 2013b):

- Enhanced dissolution rate
- Increased solubility
- Increased adhesion to surface

As the size of the particles decreases, the surface-to-volume ratio increases which enhances dissolution rate. Relation between surface area/diffusion layer thickness and dissolution rate is expressed by Noyes-Whitney equation (Mosharraf & Nyström, 1995), Equation 1, where m denotes mass of dissolved material, t time, A surface area of particles, D diffusion coefficient, d thickness of boundary layer, C_s supersaturated concentration and finally C_b denotes concentration in solution. Increased surface (A) is available for interactions with surrounding solvent, which leads to increase in mass of dissolved material in time (dm/dt), in solubility and dissolution rate. Also with nanoscale particles the thickness of the diffusion layer is lower as compared to micron sized particles.

$$\frac{dm}{dt} = A\frac{D}{d}\,(C_s - C_b)$$

Equation 1: Noyes-Whitney equation for calculation of the dissolution rate. dm/dt stands for the increase in mass of dissolved material in time, A stands for surface area of particles, D *for diffusion coefficient,* d *for thickness of boundary layer,* C_s *for supersaturated concentration and finally* C_b *denotes concentration in solution.*

Additionally, the reduction of particle size to nanoscale facilitates solubility increase. It is presented by the Ostwald-Freundlich equation (Bentley, 1977), Equation 2, where ratio between solubility of a particle S_r and solubility of bulk material S_∞ increases exponentially according to radius r of a particle. This law also describes influence of other properties of particle material, γ denotes surface energy of material, ρ and M denotes respectively its density and molecular weight. This equation takes into account a significant influence of temperature denoted as T and its relation to energy scale represented as gas constant R (8,314 J mol^{-1} K^{-1}).

$$\ln \frac{S_r}{S_\infty} = \frac{2\,\gamma}{\rho\, r} \frac{M}{R\, T}$$

Equation 2: Oswald-Freundlich equation for calculation of solubility changes according to particle size. S_r stands for solubility of a particle, S_∞ for solubility of bulk material, r for radius of a particle, γ for surface energy of material, ρ for density of the material and M for molecular weight. T stands for temperature and R for gas constant (8,314 J mol^{-1} K^{-1})

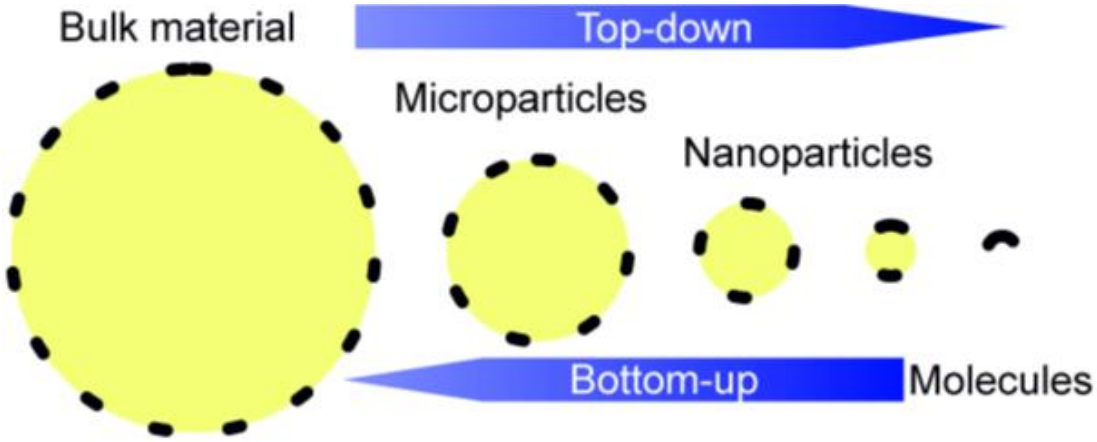
The ability of nanocrystals to adhere to mucosa of digestive tract is beneficial, as well. The contact time is prolonged and thereby the absorption is promoted (Ponchel, Montisci, Dembri, Durrer, & Duchêne, 1997). The total surface increases, so larger area is available for interactions.

Previous study indicates that all three items are combined to obtain *in vivo* higher bioavailability (Gao et al., 2012). Enhanced dissolution rate enables faster absorption, T_{max} is also achieved faster and C_{max} values are higher. However, the key aspect in improving of drug absorption is a supersaturated solution due to increased solubility and accelerated dissolution rate.

3.3 Production techniques

The production of nanoparticles can be divided into two classes, either bottom-up or top-down techniques (Peltonen, Hirvonen, & Laaksonen, 2013c).The formation by bottom-up technique means building the nanoparticles up from predissolved molecules, such as antisolvent precipitation or liquid atomization based techniques. Top-down methods start with bulky material which is then broken down to

nanoparticles, for instance by wet ball milling or high-pressure homogenisation techniques. Moreover, above mentioned techniques can be combined to shorten the production time or in order to reach smaller particles. The summary of the techniques is depicted in Figure 2.



Top-down methods

Pearl milling
High pressure homogenization
Combination methods
(e.g. pre-milling + HPH)

+ Simple and fast
+ Repeatibility
+ Easy scale-up
- Contamination
- Energy intensive

Bottom-up methods

Anti-solvent precipitation
Electrospray
Aerosol flow reactor

+ Small size
+ Monodispersity
- Scale-up
- Finding suitable conditions takes time
- Controlling particle growth
- Solvent residues

Figure 2. Summary of different nanoparticle production techniques including their advantages and disadvantages. Adopted from Handbook of Nanobiomedical Research, Chapter 5

In general, formulation of drugs is struggling with quantum of added excipients and their possible toxic effects on human body. Simple structure of nanocrystals, consisting from solid drug core and covering stabiliser layer, often overcomes this problem. Production is facilitated, because only stabiliser, or combination of stabilisers, is required for it.

3.3.1 Bottom – up techniques

Mostly utilized bottom-up technique is antisolvent precipitation method. With this technique, drug and stabiliser are first dissolved in a solvent. When antisolvent is added, the solubility of the drug decreases rapidly. It is necessary to find a suitable stabiliser in order to form appropriate mixture. This precipitation is usually supposed to run fast, nevertheless, not only the type of added antisolvent but also the conditions of the process play an important role. The right temperature and optimal drug and stabilizer concentration have to be found to form nanoparticles with narrow size deviation. A key limitation of this method is the solubility of the drug in the same solvent in which the antisolvent is soluble. Another complication, that occurs regularly, is long-running and complex solvent removal. Possible toxicity of solvent has to be taken into account, as well. In addition, it is difficult to control the particle size properly. To stop particle growth, a microfluidizer is utilised (Müller & Moschwitzer, 2006)

Another class of bottom-up techniques are liquid atomization techniques, like spray drying (Peltonen, Valo, Kolakovic, Laaksonen, & Hirvonen, 2010), electrospraying (M. Wang, Rutledge, Myerson, & Trout, 2012) and aerosol flow reactor method (Eerikäinen, Watanabe, Kauppinen, & Ahonen, 2003). Spray drying is method mostly applied to formulation of microparticles, but nanosizing is possible, as well. The solution of drug is atomised after passing through a nozzle, created droplets are dried. Electrospraying applies a high voltage to liquid in the nozzle that results in creation of small charged droplets that dry into nanoparticles. In aerosol flow reactor method droplets from precursor solution are created into carrier gas medium which forms final particles. Formed drug particles are often in amorphous form so if crystalline form is preferable, an additional steps are required to crystallize the drug, such as annealing. Liquid atomization techniques are not utilised very frequently.

3.3.2 Top-down techniques

Wet ball milling (pearl milling) is performed in vessels, which are made of and/or coated by hard ceramic material, such as zirconium oxide. The same material is used to produce the pearls. During the milling

the temperature increases and the high shear forces are induced, thus the protection of device is necessary to avoid contamination risk.

The milling pearls perform the milling. During the milling, the number of collision is judged to be a significant issue, although the collision energy is provided co-operatively with the pearl weight (Niwa, Miura, & Danjo, 2011). The size of pearls varies typically from 0,3 mm to 1 mm to achieve nanoscale particles, but also bigger pearls exist. The smaller the milling pearls, the finer the particles are produced. Pearls with diameter below 0,3 mm are not common because of more demanding separation process from the nanosuspension.

Conditions of milling have to be taken into account if considering the resultant size of the particles. Critical parameters are milling speed and time, the characteristic of milling pearls, temperature and the amount and properties of drug and stabiliser. For milling, a proper stabiliser, that reduces the risk of aggregation, is dissolved in an aqueous medium, and the solid drug is added to form a dispersion.

To prevent contamination of the milled slurry, the high milling speed (1000-4000 rpm) for short time (minutes) is preferred as compared to low speed (80-90 rpm) during long period of time (1-5 days) (Liu et al., 2011). If milling is performed at high speed, appropriate cooling might be needed during the milling. Milling and cooling periods can be repeated in order to achieve the desired particle size

Scaling-up and reproduction of milling is possible in an easy manner, therefore milling techniques are widely used for commercial products by manufacturers.

High-pressure homogenisation (HPH) by piston gap or microfluidizer are another group of techniques for top-down production of drug nanocrystals (Hao et al., 2012; Keck & Müller, 2006). The piston gap technique employs cavitation, high sheer forces, particle collision and turbulent flow to decrease the particle size. Dispersed drug in a stabiliser solution is delivered by piston through a small gap at high speed. Inside the narrow gap, dynamic pressure increases simultaneously as the static pressure is lowered below the vapour pressure. It causes water to boil within the gap. The cavitation induced by boiling, can be accomplished only in aqueous medium.

The main principle of microfluidizer is jet stream. Two liquids collide at high velocity under high pressure. Shear forces and cavitation forces are present and they cause the size reduction of dispersed drug.

In both methods, homogenization cycles are repeated until the product has acceptable properties. Multiple homogenization cycles are required to achieve the nanoscale particles.

3.4 Critical parameters of nanocrystals

Characterisation of produced nanoparticles is essential for repeatability, detailed description of formulation process, and for designing and approval of storage conditions. Particle size, shape and surface are measured, the structure of crystals and their dissolution are probed. The nanoscale influences the applicability of techniques; light scattering analysis, electron microscopic methods, thermal analysis and dissolution testing are important characterization methods (Peltonen, Hirvonen, & Laaksonen, 2013a).

3.4.1 Particle size and particle size distribution

There are several techniques to measure the particle size, for example light scattering (Nobbmann & Morfesis, 2009), transmission electron microscopy (TEM) (Abdelwahed, Degobert, & Fessi, 2006b), scanning electron microscopy (SEM), environmental scanning electron microscopy (Abdelwahed, Degobert, & Fessi, 2006a) and atomic force microscopy (Shahgaldian, Gualbert, Aïssa, & Coleman, 2003). Light scattering and electron microscopic techniques count among the most common ones.

DLS measurements the main principle is a light ray that traverses cuvette containing measured substance in a solvent. As the light is scattered from a suspension or solution, the deflections and the intensity changes of a ray are measured. According to resulting ray angle and intensity, the particle size can be derived. This method provides fast, precise and sensitive conclusions. The problem with light scattering occurs if the high polydispersity level appears. In that case, large particles are overexpressed. This complication requires size-fractionation for very heterogeneous sample before the measurement. On the contrary, mentioned pre-handling step can introduce ambiguity in the interpretation.

The measurement is influenced by the refractive indices of the particles and the medium, Brownian motion has to be taken into consideration, as well. Measurement results are approximated to spherical shape of particles, thus for example a needle-like shape can cause misinterpretation. Therefore, the confirmation of results, by SEM for example, is recommended.

The main advantage of electron microscopy techniques lies in simultaneous measurement of structure and shape of a sample. SEM scans the sample in a raster with a beam of electrons, whose interaction with a sample surface leads to various signals that can be detected. The coating of the sample with platinum or some other material may be needed, which could influence the sample properties (Ito, Sun, Bevan, & Crooks, 2004). Also vacuum and the electron beam can have an impact on a sample, and the analysis are time consuming.

3.4.2 Morphology

As mentioned above, the electron microscopic techniques are a useful tool to probe the morphology of nanoparticles. To measure surface area, gas adsorption based on the Brunauer, Emmett and Teller (BET) technique can be used (Hausberger & DeLuca, 1995). Liquid nitrogen is absorbed onto the particle surface. A monolayer, that is formed, determinates the surface area, in case of porous materials also mean pore size and pore size distribution can be measured.

3.4.3 Chemical and solid state analysis

The main approach in determination of chemical composition is X-ray photoelectron spectroscopy (XPS). To characterize the physical structure and properties of the material differential scanning calorimetry (DSC), X-ray diffraction (XRD) or variable temperature X-ray diffraction (VT-XRD) (Peltonen, Koistinen, Karjalainen, Häkkinen, & Hirvonen, 2002) that is complementary to DSC can be used. To recognise specific functional groups in structure, infrared spectrophotometer (IR, FT-IR) (Moon, Urban, & Milliron, 2009)and NMR spectroscopy (Gomez, Guerra, Myers, Crooks, & Velders, 2009) are the preferential methods.

Differential scanning calorimetry (DSC) describes thermal behaviour of the sample. It measures the endothermic or exothermic changes between heated sample and reference. The difference is recorded as a function of temperature.

Weight of sample, reference and both aluminium pans with lids influence the amount of required heat energy as well, therefore for quantitative analysis these parameters must be known accurately. The outcome is a thermogram that describes glass transition temperature, melting point, polymorphic changes, recrystallization etc. This characteristics are unique for each substance. This fact is utilised to probe whether any unwanted changes in chemical composition or in solid state have occurred during the production phase. If the characteristics of each substance remain unchanged within a mixture, chemical and physical changes can be excluded. Previous research has documented the significant role of this method (Hyvönen, Peltonen, Karjalainen, & Hirvonen, 2005; Y. Wang et al., 2012).

3.4.4 Dissolution and solubility testing

Solubility testing in nanoscale requires sufficient sensitivity due to high speed of dissolution (Peltonen, Hirvonen, & Laaksonen, 2013d) .

To quantify the amount of dissolved drug, mostly high-performance liquid chromatography (HPLC) and UV spectroscopy are employed. HPLC as a separation method confirms the structure of drug and quantifies the drug amount. On the other hand, this method is time-demanding and more expensive compared to UV spectroscopy. UV spectroscopy is a common technique based on light absorption. Measurements are performed easily and more samples can be measured after each other in short time.

After taking a sample, prior to determination of the amount of dissolved drug, filtration and ultracentrifugation are the most common methods to separate remaining particles. In both of them, the errors might appear. In filtration, mostly a 0,1 – 0,45 μm filter is used. However, there is probability that small particles pass through the filter. Small particles influence later the techniques employed to measure the concentration of dissolved drug. For example, particles dissolve later in an HPLC mobile phase or influence transparency of sample measured by UV spectroscopy. In both cases, the results are overestimated. Also interactions between the filtrate and the filter material affect the result. Ultracentrifugation is based on sedimentation of denser particles that is accelerated. Owing to the small size and low density of nanoparticles,

the ultracentrifugation takes prolonged time to be performed. During this period, the changes in dissolution can occur.

Therefore, *in situ* dissolution methods have been developed. *In situ* dissolution methods enable analysis of the sample directly, inside the dissolution vessels, so errors occurring in separation methods are avoided. Despite the novel approach, the use of *in situ* methods is restricted to certain group of samples. It stands for UV method that are based on light scattering and absorption of light by the nanoparticles and thus prove their presence (Van Eerdenbrugh, Alonzo, & Taylor, 2011). The limitation of electrochemical in situ techniques lies in necessity of charged molecules, but the majority of poor water soluble drugs are uncharged (Mora et al., 2009). Among other methods counts calorimetric measurement (Kayaert et al., 2010) or turbidimetric measurement in which the turbidity, and thus the solubility, of the nanosuspension alters depending on the amount of added surfactant (Crisp, Tucker, Rogers, Williams, & Johnston, 2007). Another promising method that has been recently reported is a variation of microdialysis, the pulsatile microdialysis method (Shah, Patel, Khairuzzaman, & Bellantone, 2014).

3.5 Stabilisation of drug nanocrystals

Stabilisers are compounds, mostly polymers or surfactants, used to eliminate the major drawback of nanocrystals – physical instability. Owing to nanosize the particles tend to aggregate, there is a risk of Oswald ripening and the changes in polymorphic form can occur, as well. The changes are the most common during production or long-term storage (Sharma, Denny, & Garg, 2009). The formulation of the nanosuspension without a stabiliser is an exception because the stabiliser plays a vital role, mainly for the smallest particles.

The stability problem occurs because of increase of free energy during production. Free energy (*ΔG)* arises when a larger surface area (*ΔA*) is formed. Equation 3 that describes this law is as follows:

$$\Delta G = \gamma_{s/l} \times \Delta A$$

Equation 3: Equation for calculation of free energy. ΔG stands for free energy, $\gamma_{s/l}$ for interfacial tension, ΔA for surface area.

In this equation $\gamma_{s/l}$ denotes interfacial tension. The system tends to reduce this high-energetic state, either by dissolution of nanoparticles or by aggregation. Dissolution creates solution and generally solutions are more chaotic systems and thus are more stable. Of course with solutions to this problem can be the precipitation back to low energy solid form. By forming aggregates, both surface area and consequently free energy decrease. To prevent reduction of high-energetic state by system itself, stabilisers are added. The majority have a surface active properties which lower surface tension and, hence, according to the equation, also free energy of the system (Rabinow, 2004).

Usually amphiphilic compounds function as surfactants. The hydrophobic part of molecule is attached to hydrophobic drug and the hydrophilic chain faces towards water molecules. This process decreases interfacial tension and facilitates the wetting of the hydrophobic drug. In addition, a steric barrier is formed between neighbouring nanoparticle surfaces, and thus their interactions are minimized. The efficient molecular weight of polymers building a steric hindrance ranges from 5 000 to 25 000 g/mol. If stabilisation is based purely on electrostatic forces, then the zeta potential values below -30 mV or above +30 mV are necessary. Steric stabilisation is more thermolabile, thus drying has to be done carefully. Electrostatic stabilisation is sensitive to pH changes, drying and changes in ionic composition of medium (Peltonen, Hirvonen, & Laaksonen, 2013e).

Numerous polymers, widely used in pharmacy, are suitable to stabilise the nanocrystals, such as cellulose derivatives, PVP (Van Eerdenbrugh et al., 2009) and poloxamers (Liu et al., 2011). Also simple surface-active agents, for example D-a-tocopherol polyethylene glycol 1000 succinate (TPGS), sodium dodecyl sulfate (SDS) or Tweens, can provide sufficient protection from aggregation. Regarding small surfactant molecules, there is a risk of solubilisation of the drug inside surfactant micelles, which would disrupt the nanocrystal formation. The choice of stabiliser depends on the drug and production technique, for instance, in milling it is necessary to protect already newly created nanocrystals from aggregation in comparison with end-product stabilisation mainly needed in case of HPH.

Moreover, the amount of stabiliser has to be optimized. If the amount of stabiliser is not sufficient, the nanocrystals may not be formed (Peltonen et al., 2013e).

On the other hand, an excess of stabiliser might lead to enhanced solubility and Ostwald ripening appears. Also, extra stabiliser may form sticky layers which facilitate aggregation. The optimal amount of stabiliser is characteristic for each drug-stabiliser pair, but usually it varies from 5 % to 50 % of the drug amount.

Another property of stabiliser, that needs to be taken into account, is viscosity (Van Eerdenbrugh et al., 2009). High viscosity is a disadvantageous fact if wet milling or HPH is employed. It decelerates the preparation process, increases the energetic demands, and impedes separation in case of wet milling. On the other hand, higher viscosity stabilises system, hinders aggregation and positively influences maintenance of supersaturated solution. Therefore, the amount of high viscose polymer has to be considered reasonably, added amount is mostly lower (10 %) (Van Eerdenbrugh et al., 2009)

Generally, the addition of stabiliser takes place before starting of the nanocrystallisation procedure, but the stabiliser can be added periodically during the nanocrystallisation process, as well (Bhakay, Merwade, Bilgili, & Dave, 2011). The periodic addition has a positive impact on diminishing the particle size and narrowing its deviation. Adding a high-viscous polymer in parts has been shown beneficial, because the increase in viscosity is gradual.

3.5.1 Poloxamer 407

Poloxamers are block copolymers. Poloxamer 407 (trade name Pluronic F 127) (Rowe, Sheskey, Cook, & Fenton, 2012a) consists of polyethylene oxide (PEO) located peripherally, creating hydrophilic parts of molecule, and central polypropylene oxide (PPO) forms hydrophobic chain (Figure 3). The structure can be summarized as ABA, where A stands for PEO and B for PPO. Poloxamers are a typical example of amphiphilic stabiliser. The hydrophobic chains adhere to the hydrophobic drug crystals surface while the hydrophilic part of molecule is attached to aqueous medium and forms a steric barrier against aggregation. The length of hydrophilic tails is important for steric stabilisation in order to provide adequate steric hindrance.

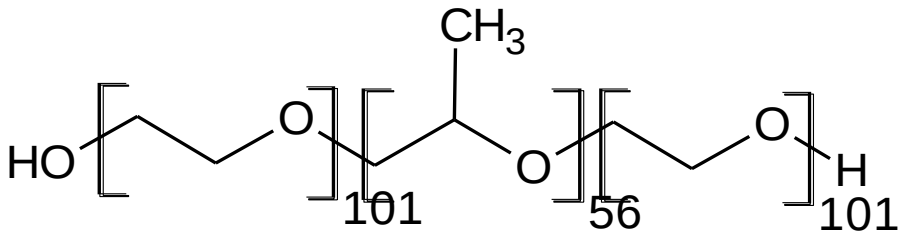


Figure 3: Molecular structure of Poloxamer 407 (F 127)

Poloxamer 407 is a solid stabiliser that is freely soluble in water, ethanol 95% and propylene glycol (Figure 3). The aqueous solubility depends on temperature, with rising temperature solubility diminishes. Viscosity and surface tension values of different poloxamers are functions of molecular weight.

Table 2. Chemical properties of poloxamer 407 (F127

Property	**Poloxamer 407**	**Reference**
Molecular weight	9 840 – 14 600 g/mol	(*European Pharmacopoeia*, 2014b)
Melting temperature	56℃	(BASF The Chemical Company, 2010)
Viscosity	3 100 mPa.s	(BASF The Chemical Company, 2010)
Surface tension (0,1 % sol. 25℃)	41 mN/m	(BASF The Chemical Company, 2010)
Relative amount of PEO	71,5 - 74,9 %	
Number of PEO units	202	
Number of PPO units	56	
Solubility in water	Very soluble (less than 1 ml of solute)	(*European Pharmacopoeia*, 2014b)
Solubility in ethanol 96%v/v	Very soluble (less than 1 ml of solute)	(*European Pharmacopoeia*, 2014b)

3.6 Supersaturated state

As mentioned in chapter 3.1.13.1.1 Methods improving water solubility, multiple options to increase the solubility have been created. However, the key point is to maintain the solubility at the appropriate level until absorption takes place.

A saturated solution is defined as a solution in which the number of molecules dissolving from solid solute is equal to the number of molecules precipitating back

from the solution; thermodynamic equilibrium solubility is reached. Supersaturated state is state rich in dissolved material, where the solubility raises above thermodynamic equilibrium solubility; in supersaturated state the dissolution properties of poorly soluble materials are improved. Supersaturated state also creates a higher concentration gradient over the cell layer and thus may facilitate passive diffusion in the GI tract and improve permeability leading to higher amount of drug absorbed. However, as a state exceeding equilibrium, it tends to return to the most stable state, to the equilibrium state. This thermodynamic instability leads to precipitation. When precipitation/crystallisation occurs, concentration decreases rapidly and advantage of concentration gradient is lost. Figure 4 shows changes in concentration according to the level of saturation.

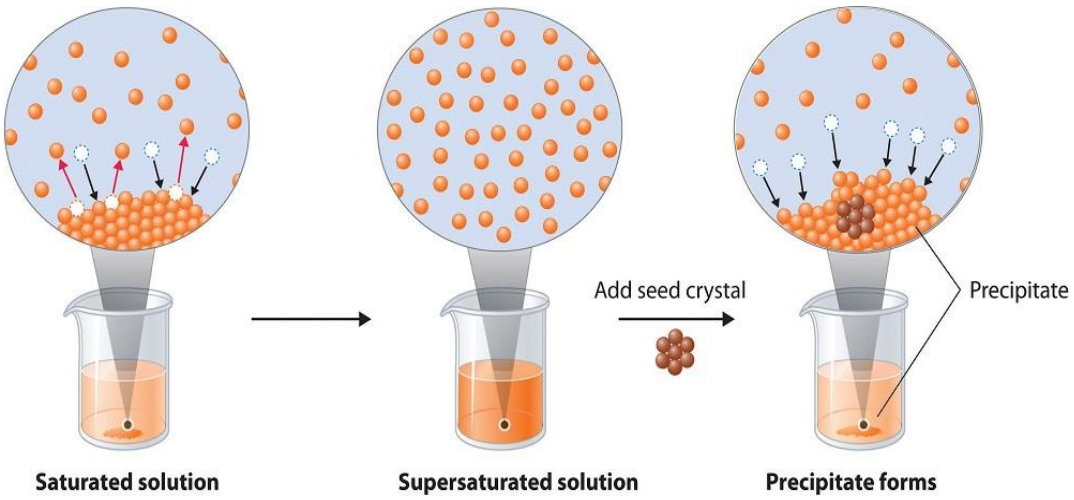


Figure 4. Saturated solution, supersaturated solution and precipitation. Thermodynamic equilibrium in saturated solution is depicted by the same length of arrows that denote migration of molecules. If precipitation occurs, molecules migrate from the solution to solid form. Concentration changes are illustrated by different shades of orange of liquid in the beaker. Modified from http://chemwiki.ucdavis.edu/

Precipitation is an unavoidable phase separation that occurs to return the thermodynamic system to equilibrium. The precipitation process proceeds via two steps – nucleation and crystal growth. The nucleation can be initiated on a surface of impurity or seeds are required. Arisen structures gather solute molecules and form clusters. Clusters under critical size can be dissolved, however, after reaching a sufficient size, crystal growth follows by attaching more of dissolved solute molecules.

The rate of nucleation and crystal growth is accelerated by the intrinsic parameters of the solution, such as increasing degree of supersaturation and low medium viscosity. External conditions have impact as well, for example presence of impurities in solution or lower temperature (Warren et al., 2010).

In order to inhibit the mentioned phase separation, precipitation inhibitors are added to stabilise the supersaturated solution. The aim is to delay this phase separation until the absorption area is reached. There are two main mechanisms of action that participate in the maintenance of supersaturation – inhibition of precipitation and co-solvency (Warren et al., 2010). Precipitation can be inhibited at the nucleation or precipitate growth level. Stabilisers stay in intimate contact with a poorly water soluble drug and stabilise its dissolved state via intermolecular interactions. From these low energy chemical bonds, hydrogen bonding and hydrophobic interactions play a major role (DiNunzio et al., 2008). Co-solvency is typical for surfactants and its function consists of an increase of equilibrium solubility that diminishes the degree of supersaturation, thus lowering thermodynamic forces that induce the return to the stable saturated state. Several parameters of stabiliser influence its effect on inhibition of precipitation, such as molecular weight, viscosity, and amount of function groups enabling hydrogen bonding (Warren et al., 2010). Higher molecular weight signifies longer chains with more functional groups to bind the poorly soluble drug and support drug-stabiliser interactions. The viscosity value influences the ability of drug to diffuse throughout the aqueous medium. The higher the viscosity is, the more restricted the diffusion and thus the gathering of dissolved molecules is. Hydrogen bonds ensure closer attachment of stabiliser and drug and thus the stabiliser can inhibit precipitation.

Several publications have appeared in recent years documenting the importance of this issue. Precipitation inhibitors, such as hydroxypropylmethylcellulose (HPMC), polyvinyl pyrrolidone (PVP) and hydroxypropylmethylcellulose acetate (HPMCAS), have been utilized to stabilise supersaturated state (Konno et al., 2008; D. a Miller, DiNunzio, Yang, McGinity, & Williams, 2008). Most of the studies have focused on maintaining the supersaturated state after dissolution of amorphous drugs. Nevertheless, *Ueda et al.* indicated the inhibition of carbamazepine precipitation by stabilised nanoparticles (Ueda et al., 2015). In the study supersaturated state of sample that contains stabilised nanoparticles

persists longer compared to the temporary supersaturation provided by amorphization. Figure 5 displays different shapes of dissolution curves influenced by the presence of properly stabilised nanoparticles. So called parachute effect prolongs the high level of solubility reached at the beginning and thus is desired to maintain the supersaturated state.

Recent studies have also revealed that not only the type of precipitation inhibitor, but also the stage of manufacturing procedure and physical phase (solid material, inhibitor in solution), influence the final effect on stability of supersaturated solution (Surwase et al., 2015).

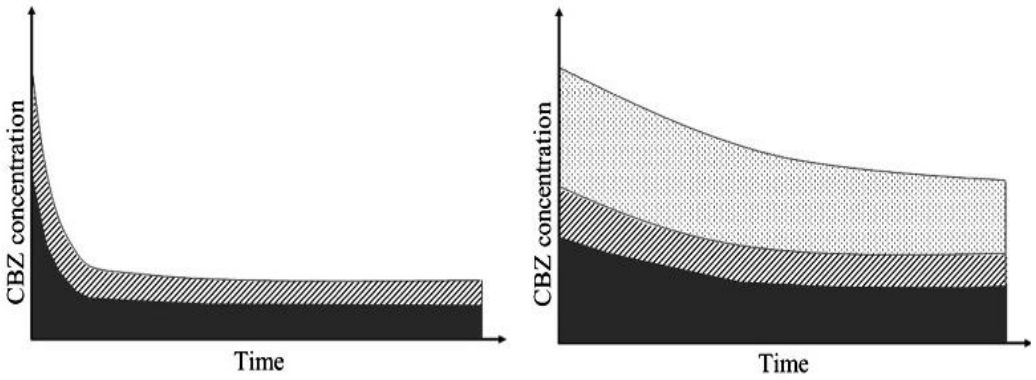


Figure 5: Different shape of dissolution curves. The high initial peak reaching temporary supersaturation in case of amorphization in the first graph compared to maintained supersaturation state in presence of stabilised nanoparticles in the second graph where the parachute effect can be observed. Various shades of grey denote states and particles sizes of the composition, the brightest denotes the presence of nanoparticles.
CBZ –carbamazepine
Modified from (Ueda et al., 2015)

3.6.1 Stabilisers of supersaturated state

3.6.1.1 *Hydroxypropyl methylcellulose/hypromellose*

HPMC is a semisynthetic, non-ionic, inert polymer, chemically it is a cellulose ether that is created by substitution of methoxy and hydroxypropyl groups (Figure 6).

The extent of substitution varies in hydroxypropyl and methyl content, 4-12 % and approximately 30 %, respectively. The degree of substitution gives a different physicochemical properties to particular hypromellose grades.

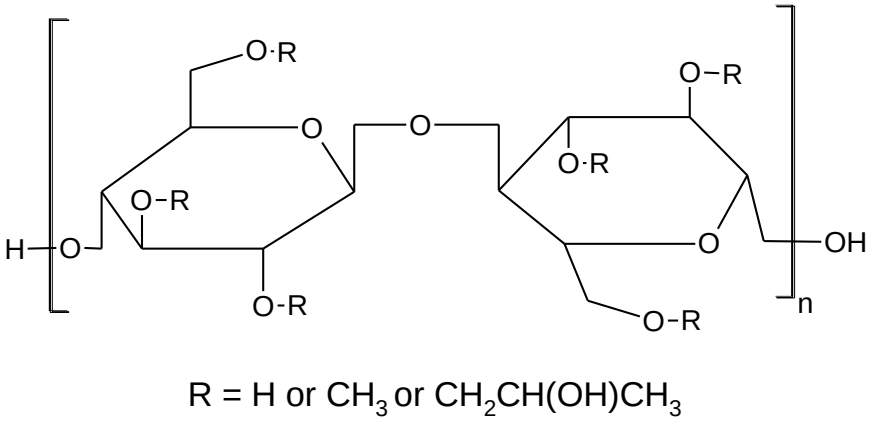


Figure 6. Molecular structure of hydroxypropyl methylcellulose

Hypromellose is a stable powder, which is soluble in cold water, but insoluble in hot water and organic solvents (Figure 6). Hypromellose is good stabiliser for nanocrystals, though quite high viscosity of HPMC solutions complicates the preparation of nanoparticles. The viscosity depends on the degree of substitution and concentration. The temperature is an important aspect that influences the sol-gel transformation. The gelation temperature varies from 50℃ to 90℃ and it is related to the grade of methoxy group and concentration of solution. For temperatures below the gelation temperature as the temperature increases, the viscosity decreases. Beyond the gelation temperature the viscosity is directly proportional to the temperature. Also acid-base properties contribute to the behaviour of HPMC. Hypromellose acts as proton donor due to its free hydroxyl groups. In aqueous medium it produces a proton-rich micro-environment that enables the ionization of ITZ and accelerates the dissolution rate (D. a Miller et al., 2008). Referring to acid-basic properties, pH of medium must be taken into account, as well.

Table 3. Chemical properties of hydroxypropyl methyl cellulose

Property	**Methocel E5 Premium LV EP, type 2910 (LV = low viscosity, EP= meets European Pharmacopeia requirements)**	**Reference**
Viscosity (2% w/v)	4-6 mPa.s	(The Dow Chemical Company, 2002)
Molecular weight	11 000 – 12 000 g/mol	(The Dow Chemical Company, 2002)
Methoxyl %	28-30 %	(The Dow Chemical Company, 2002)
Hydroxypropyl %	7-12 %	(The Dow Chemical Company, 2002)
Solubility in cold water	Dissolves giving a colloidal solution	(*European Pharmacopoeia*, 2014c)
Solubility in hot water	Practically insoluble (more than 10 000 ml per gram of solute)	(*European Pharmacopoeia*, 2014c)
Solubility in organic solvents (acetone, anhydrous ethanol, toluene)	Practically insoluble (more than 10 000 ml per gram of solute)	(*European Pharmacopoeia*, 2014c)

3.6.1.2 *Polyvinylpyrrolidone / povidone*

Polyvinylpyrrolidone is a synthetic, non-ionic, physiologically inert polymer consisting of N-vinylpyrrolidone monomers with a linear backbone (Rowe, Sheskey, Cook, & Fenton, 2012b) (Figure 7).

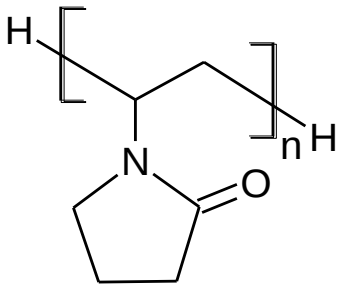


Figure 7. Chemical structure of polyvinylpyrrolidone

Different PVP grades can be found based on the molecular weight. K-value describes the properties of the PVP grade; this value is calculated from the equation (Equation 4) that considers relative viscosity of the solution of certain concentration (Rowe et al., 2012b).

$$\log z = c\left[\frac{75\,k^2}{1+1{,}5kc}\right] + k$$

Equation 4: Fikentscher´s equation for calculation of K-value. z stands for relative viscosity of the solution, c for concentration of the solution, k for K-value×10^{-3}

PVP is well soluble in cold water, but with higher concentrations viscosity restricts the solubility (Table 4). The solubility in organic solvents varies and is related to K-value. Povidone is an amphiphilic polymer with ability to accept protons, thus it builds complexes with other molecules, mainly with H-donors, for example phenols or carboxylic acids. Therefore, there is a competition between weak basic ITZ and PVP that might influence dissolution rate (D. a Miller et al., 2008), with respect to pH of the medium. Viscosity depends on concentration and molecular weight and is included in K-value. Up to 10 % concentrations, the viscosity is hardly affected by temperature, but increasing temperature decreases viscosity of more concentrated solutions.

Table 4. Chemical properties of polyvinylpyrrolidone

Property	**Kollidon 30**	**Reference**
pH (5% in water)	3-5	(BASF The Chemical Company, 2008)
K-value	28-32	(BASF The Chemical Company, 2008)
Typical viscosity range (10 % (g/ml) in water at 20 °C)	5,5 – 8,5 mPa.s	(BASF The Chemical Company, 2008)
Average molecular weight	44 000 – 54 000 g/mol	(BASF The Chemical Company, 2008)
Solubility in water	Freely soluble (from 1 to 10 ml per gram of solute)	(*European Pharmacopoeia*, 2014d)
Solubility in ethanol (96 %), methanol	Freely soluble (from 1 to 10 ml per gram of solute)	(*European Pharmacopoeia*, 2014d)

4 Experimental section

4.1 Materials

Itraconazole (Orion Pharma, Espoo, Finland) was used as the model drug. As stabilizers were used Poloxamer 407 (Lutrol F127) from BASF Co. (Ludwigshafen, Germany), hydroxypropyl methylcellulose (Methocel E5 Premium LV EP, type 2910) from The Dow Chemical Company (Midland, Michigan, USA), and polyvinylpyrrolidone (Kollidon K30) from BASF Co. (Ludwigshafen, Germany). Hydrochlorid acid (37%, Riedel-de Haen, Seelze, Germany) and potassium chloride (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) were used to prepare hydrochloric acid buffer (pH 1,2). Methanol (Methanol anhydrous 99,8 %, Aldrich-Sigma, Steinheim, Germany) was used to create calibration curve for measuring itraconazole concentration utilising UV spectrometry. Water used was ultrapurified Millipore® water (Millipore, Molsheim, France).

4.2 Methods

4.2.1 Media milling

The nanosuspensions were prepared using a wet-milling technique.

First, aqueous stabilizer solution was prepared by dissolving stabilizer(s) in water and shaking the solution on orbital shaker overnight.

Next, 3 ml of aqueous stabilizer solution were added to 1 g of bulk itraconazole and mixed firmly in a beaker. The obtained itraconazole suspension was inserted in milling vessels over the milling beads. Additional 2 ml of stabilizer solution were added to collect the residual suspension from the beaker to milling vessel. For milling vessel with the volume of 20 ml, 30 g of milling pearls (diameter 1 mm, zirconium oxide) were used. When higher yields were required, larger milling vessel were used, specifically milling vessel with volume of 45 ml in combination with 70 g of milling pearls (diameter 1 mm, zirconium oxide). In this case, 2 g of bulk itraconazole were mixed with 5ml of stabilizer solution and afterwards 5 ml of stabilizer solution were added to collect residual suspension.

A planetary ball mill (Pulverisette 7 Premium, Fritsch Co., Idar-Oberstein, Germany) was used for the wet milling. Grinding was performed at 1100 rpm during

3 min cycles. After each cycle the milling vessel was submerged to ice bath in order to avoid warming up of the sample. In total, 10 milling and cooling cycles were performed. The rotational direction was not reversed in milling.

Subsequent to the milling, the nanosuspension was collected by pipetting or the more viscous samples were sieved in order to separate the milling pearls from the nanosuspension. If needed the samples were dried in drying oven Cooling Incubator KBK 4330 (Ehret, Emmendingen, Germany) at 40 °C overnight.

4.2.2 Differential scanning calorimetry (DSC)

To analyse thermal behaviour and to exclude chemical interactions, dried samples were tested with DSC 832^e (Mettler Toledo Inc., Columbus, USA). A powder sample was placed in an aluminium pan, the optimal sample weight was 5 mg. The pan was covered by pierced aluminium lid and the whole pan was sealed. The temperature range was set from 25 ℃ to 200 C, with the heating rate of 10 ℃/min. The measurements were performed under nitrogen flow of 50 ml/min (Liu et al., 2011). Thermogram of pure drug and pure stabilizers, and their physical mixture in corresponding ratios, were measured as a control. The data was analysed with STAR^e v 9.00 software (Mettler Toledo, Columbus, USA).

4.2.3 Dynamic light scattering

DLS also known as photon correlation spectroscopy (PCS), was used for determining the mean particle size and polydispersity index (PDI). The measurements were performed on Malvern Zetasizer Nano-ZS (Malvern Instrument, Malvern, UK).

For analysing the mean particle size and PI, the preparation of a saturated aqueous drug solution was used. Saturated solution is utilised as a medium to dilute the nanosuspension to opalescent dispersion, saturation is necessary to prevent nanoparticles from dissolving.

The saturated solution was prepared a day before the measurements. The saturated solution contains 0,1 g of each stabilizer and excess amount of itraconazole in order to achieve saturation. All components were dissolved in 100 ml milli-Q-water and placed into orbital shaker for 24 hours. Prior to use, the saturated solution was filtered through 0,45 µm Acrodisc® Syringe filter with GHP Membrane (Pall Corporation, New York, USA).

For particle size measurement with DLS the nanosuspension sample was diluted with saturated drug solution. The dilution consists of 0,1 g nanosuspension and 1,9 ml saturated solution, which preparation is described in previous paragraph. Diluted nanosuspension was sonicated for 1,5 min. This basic 20 times dilution serves as a starting point for further dilutions, when necessary.

The sample used for the measurement was prepared from basic 20 times dilution and saturated solution. 100 µl of dilution was taken, 2 ml of saturated solution were added. If dilution of sample was not sufficient, 3 ml of saturated solution were added. The opalescent samples were shaken prior to measurement. 1ml of this sample was pipetted into the cuvette and the measurement was performed.

4.2.4 Solubility and supersaturation maintenance testing

Solubility and supersaturation maintenance testing was performed with dried powder sample at pH 1,2 at laboratory temperature (app. 25 ℃). Dissolution study samples were tested for quantitative determination of ITZ (Parikh, Patel, Dave, Patel, & Sen, 2011) using UV spectrophotometric method (UV-1600Pc Spectrophotometer, VWR International BVBA, Leuven, Belgium) . Results were evaluated using software M. Wave Professional v 1.0.

A dried powder sample was placed at the bottom of the flask. Based on addition of stabilizer at different stages of formulation, the composition of sample varied at this point. Following options were applied (summarised in Table 5):

A. all necessary stabilizers added before milling – approximately 2 mg of dried nanosuspension
B. one stabilizer added before milling - approximately 2 mg of dried nanosuspension + correspondent percentage of drug amount of the second stabilizer added in solid state to create physical mixture of two solid powders before the addition of solvent
C. one stabilizer added before milling - approximately 2 mg of dried nanosuspension + correspondent percentage of drug amount of the second stabilizer predissolved in hydrochloric acid buffer (pH 1.2)

Table 5. Various stages at which stabilizers are added and various physical states

	1.stabilizer addition stage	**2. stabilizer addition stage**
A	before the milling	Before the milling
B	before the milling	Solid powder before the solubility testing
C	before the milling	Predissolved in Hydrochloric Acid (pH 1.2)

Subsequent to addition of all the stabilizers, 20 ml of hydrochloric acid buffer (pH 1.2) were added. Hydrochloric acid buffer was prepared according to US Pharmacopoeia (*The United States Pharmacopeia (USP) 23 - National Formulary (NF) 18*, 1994).

Amount of utilised stabiliser is reported as percentage of drug amount throughout this paper.

Calibration curve was measured from a series of standards that had been prepared from a stock solution of ITZ of concentration 200 μm/ml. Due to poor aqueous solubility of ITZ methanol was chosen as a solvent. Concentrations of standards ranged from 0,2 to 15 μm/ml, specifically 0,2; 1; 2; 5; and 15 μm/ml. Each concentration was prepared in duplicate. Calibration curve was recorded at wavelength of 262 nm. The calibration curve is represented by following equation: $c=14,8238A+0,5457$, $r^2=0,987293$ where *c* stands for concentration, *A* for absorbance and *r* for correlation coefficient.

Sample aliquots (~3ml) were taken after 5, 15, 30, 60, 120, 240, 360 and 1440 minutes after starting the solubility testing, filtered and analysed. Subsequent to sampling, same amount of fresh hydrochloric acid buffer (3ml) was added to dissolution flask to maintain the volume of the medium. For filtration 0,2 μm Acrodisc® Syringe filter with GHP Membrane or Acrodisc® Syringe filter 0,2 μm Supor R® Membrane was utilized. Subsamples derived from 0,2 F127 and bulk samples were filtered by Acrodisc® Syringe filter 0,8 μm Supor R® Membrane The filtrate was clear upon visual inspection.

The filtrate was inserted into cuvette and UV spectroscopy was performed. Measurements were recorded at wavelength of 262 nm.

5 Results and discussion

5.1 Evaluation of chemical and physical stability

To exclude chemical or physical changes, during wet milling and drying, for example changes in crystallinity or crystal form, DSC was chosen as a technique to analyse sample composition. The experiment was carried out with two samples containing different stabiliser combination – 0,7 F127+0,1 HPMC and 0,7 F127+0,1 PVP. Obtained thermograms were compared to corresponding curves of pure substances and physical mixture.

Melting point of ITZ is at app. 166 °C (O'Neil, 2006), melting peak of F127 can be found at 54 °C (BASF The Chemical Company, 2010).

Figure 8 depicts thermograms of sample 0,7 F127+0,1 HPMC, pure bulk ITZ, pure stabilisers and a physical mixture created from bulk material with composition corresponding to sample 0,7 F127+0,1 HPMC. In HPMC thermogram an endothermic event occurs. It is located approximately at temperature 80 °C. At this temperature HPMC loses bound water molecules which causes higher heat energy consumption. In Figure 9 thermograms of sample 0,7 F127+0,1 PVP and corresponding pure materials and physical mixture are displayed. PVP curve shows an endothermic event at temperature app. 100 °C. At this temperature, water molecules are detached from polymer structure. Unlike HPMC, PVP is a hygroscopic polymer, thus tightly bound water is detached at higher temperature. In both milled nanosuspensions and corresponding physical mixtures, slight shifts of ITZ and F127 melting peaks to lower temperature are to be found. Peaks present in milled samples are slightly wider than the ones in physical mixture. This may be reasoned by nanoscale of milled particles.

Based on presence of drug melting peaks in thermogram of nanosuspension, the crystalline state of milled nanosized drug can be confirmed. Considerable differences between samples and physical mixtures cannot be found, thus, it can be assumed that no chemical or physical changes arises during wet milling and drying.

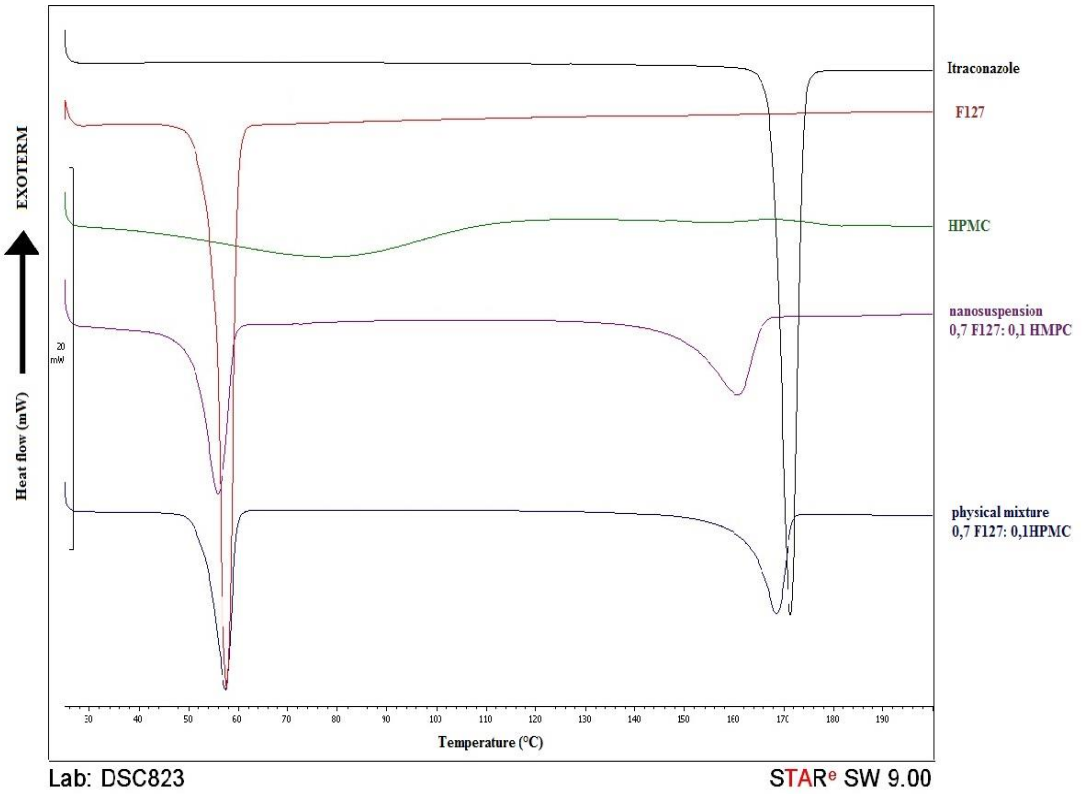


Figure 8. Differential scanning calorimetry analysis (DSC) of nanosuspension 0,7 F127:0,1 HPMC , physical mixture of the same composition, pure bulk drug (itraconazole), and stabilisers

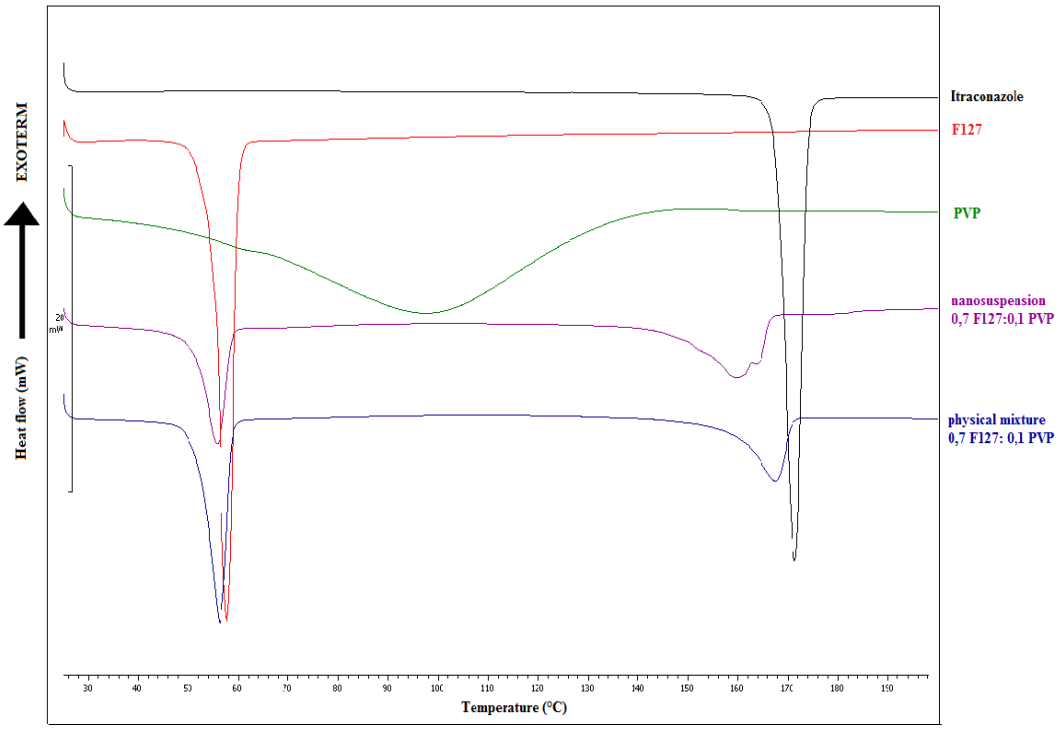


Figure 9: Differential scanning calorimetry analysis (DSC) of nanosuspension 0,7 F127:0,1 PVP , physical mixture of the same composition, pure bulk drug (itraconazole), and stabilisers

5.2 Effect of stabilisers on particle size

Next, the effect of selection of a single stabiliser or a combination of stabilisers was employed for stabilization of nanocrystals. Three stabilisers with two different purposes were used: HPMC and PVP, as compounds positively influencing maintenance of supersaturated state and poloxamer F127, as an agent preventing particle aggregation. In case of combining, always stabilisers from different groups were selected. As the results have confirmed that not only the choice of stabilisers, but also its concentration influences final particle size and PDI value. Amount of stabiliser added is referred as percentage of drug amount throughout whole thesis.

Size achieved by wet milling ranges from 248 nm to 1647 nm, PDI values are mostly below 0,3 which confirms low polydispersity level of the sample. Composition of stabilisers and results of particle size measurements are summarised in Table 6.

Table 6. Compositions of stabilisers, particle sizes and PDI of the nanosuspensions. Percentage data of stabiliser are related to content of ITZ in the sample.

Sample name	**Composition of stabilisers**			**Particle size (nm)**	**PDI**
	F127	**PVP**	**HPMC**		
0,6 F127: 0,2 HPMC	60 %	-	20 %	325	0,166
0,7 F127: 0,1 HPMC	70 %	-	10 %	334	0,120
0,6 F127: 0,2 PVP	60 %	20 %	-	248	0,166
0,7 F127: 0,1 PVP	70 %	10 %	-	253	0,162
0,25 PVP	-	25 %	-	780	0,600
0,1 HPMC	-	-	10 %	1647	0,203
0,4 PVP	-	40 %	-	979	0,287
0,25 HPMC	-	-	25 %	1239	0,226
0,55 F127: 0,25 HPMC	55 %	-	25 %	322	0,251
0,5 F127: 0,3 PVP	50 %	30 %	-	296	0,187
0,1 F127: 0,25 HPMC	10 %	-	25 %	382	0,165
0,2 F127: 0,25 HPMC	20 %	-	25 %	365	0,181
0,2 F127	20 %	-	-	397	0,227
0,1 F127	10 %	-	-	-	-
0,1 F127 : 0,25 PVP	10 %	25 %	-	973	0,199
0,2 F127 : 0,25 PVP	20 %	25 %	-	317	0,167

Poloxamer employs its superior steric hindrance provided by hydrophilic tails and enables formation of smaller particle sizes compared to other stabilizers used at the same concentration (0,2 F127 – 397 nm) . Nevertheless, in combination with 10 – 20 % of HPMC or PVP even smaller particles are created (0,7 F127 : 0, 1 HPMC – 334 nm, 0,6 F127 : 0,2 HPMC – 325 nm) especially the combination of Poloxamer with PVP shows size reduction under 300 nm (0,7 F17 : 0,1 PVP -253 nm, 0,6 F127: 0,2 PVP – 248 nm). This beneficial size reduction is related to the amount of F127. If its concentration decreases to 20 % of drug content, the particle size increases to app. 400 nm. Combination of Poloxamer with PVP or HPMC hinders this increase and particle size is 317 nm and 365 nm, using 0,25 PVP or 0,25 HPMC, respectively. The F127 concentration of 10 % (compared to drug amount) has been shown to be threshold – F127 has to be combined with another stabiliser to create nanosuspension, otherwise the sample is too polydisperse and particle size increases owing to possible aggregation and Ostwald ripening. However, contrary to previous more beneficial combination with PVP, at this F127 concentration smaller particles were prepared in combination with 25 % HMPC than with the same amount of PVP; 382 nm and 973 nm, respectively.

Using a single stabiliser with primary aim to maintain supersaturated state, results in particle size increases. PVP (25 and 40 %) stabilises the nanosuspension better compared to HPMC (concentration of 10 and 25 %, the amount of used HPMC is limited by viscosity). It is noteworthy that smaller amount of PVP gives rise to smaller particles and with increased concentration of PVP, particle size increases as well. On the other hand, the smaller particle size, formed only with PVP (25 %) is accompanied by higher PDI value (0,600). None of mentioned phenomena observed in PVP are not present when utilising HPMC. Higher amount of this stabiliser decreases the particle size. Nevertheless, HPMC in concentration of 10 % and 25 % do not display sufficient stabilisation to achieve the nanoscale particles – in both batches milled only with HPMC particle size exceeds 1000 nm, however, PDI values remain low.

Attached Table 7. Samples arranged in order to particle sizeTable 7 illustrates the order of samples according to the particle size.

Table 7. Samples arranged in order to particle size

Sample	particle size (nm)
0,6 F127:0,2 PVP	248
0,7 F127: 0,1 PVP	253
0,5 F127: 0,3 PVP	296
0,2 F127: 0,25 PVP	317
0,55 F127 : 0,25 HPMC	322
0,6 F127:0,2 HMPC	325
0,7 F127:0,1 HPMC	334
0,2 F127: 0,25 HPMC	365
0,1 F127: 0,25 HPMC	382
0,2 F127	397
0,25 PVP	780
0,1 F127: 0,25 PVP	973
0,4 PVP	979
0,25 HPMC	1239
0,1 HPMC	1647

Use of mixture of stabilisers is shown beneficial when aiming at diminishing of particle size. Generally, the thickness of steric barrier determinates the effectiveness of protection from aggregation. The smallest particles were achieved with the combination of F127 and PVP, both the stabilisers acting via hydrophobic interactions. In production phase ITZ is uncharged and thus more willing to form this kind of non-covalent bonds than hydrogen bonds. According to the measurements, presence of F127 is shown as a determinating factor of aggregation hindrance, higher amount of poloxamer leads to smaller particle size. Combining F127 with PVP provides the smallest particle size. Utilising HPMS, as a stabiliser empolying mostly hydrogen bindings, results in creation of bigger particles.

5.3 Effect of particle size on solubility and maintenance of supersaturated state

Following table (Table 8) arranges the samples according to concentration of dissolved drug, overall waveform and concentration in 120 min were considered as major factors. Particle sizes are listed as well.

Table 8. Solubility rate of nanocrystalline drug

Sample	particle size (nm)
0,25 HPMC	1239
0,1 HPMC	1647
0,6 F127:0,2 HMPC	325
0,25 PVP	780
0,4 PVP	979
0,7 F127:0,1 HPMC	334
0,7 F127: 0,1 PVP	253
0,55 F127 : 0,25 HPMC	322
0,6 F127:0,2 PVP	248
0,5 F127: 0,3 PVP	296
0,2 F127: 0,25 PVP	317
0,2 F127: 0,25 HPMC	365
0,1 F127: 0,25 HPMC	382
0,1 F127: 0,25 PVP	973

Generally, the curves are arranged regardless their particle size. Particle size is one of the factors that influences solubility, however, the solubility testing showed that it is not the essential parameter. Disadvantageous size does not necessarily deteminates the solubility rates because the highest peaks and also values of supersaturated solution were obtained with 0,25 HPMC and 0,1 HPMC. Particle size of these samples slightly exceeds nanoscale. This contratry fact might be explained by possible agglomeration of particles stabilise by HPMC, which is discussed more profundly in context of mechanism of action of this stabiliser at the end of the following section.

Table 9. Maximal concentration (C max) and time (T max) (time when the maximal concentration is reached), of nanosized and bulk samples.
b - bulk material

Sample	particle size (nm)	C max (µg/ml)	T max (min)
0,25 HPMC	1239	8,967	15
0,1 HPMC	1647	6,321	15
0,6 F127:0,2 HMPC	325	5,042	5
0,25 PVP	780	5,470	15
0,4 PVP	979	3,848	15
0,7 F127:0,1 HPMC	334	3,561	180
0,7 F127: 0,1 PVP	253	2,953	180
0,55 F127 : 0,25 HPMC	322	7,123	5
0,6 F127:0,2 PVP	248	2,482	15
0,5 F127: 0,3 PVP	296	2,477	180
0,2 F127: 0,25 PVP	317	3,204	5
0,2 F127: 0,25 HPMC	365	1,907	5
0,1 F127: 0,25 HPMC	382	0,983	60
0,1 F127: 0,25 PVP	973	1,100	5
Bulk ITZ	b	1,646	1355
bITZ+0,25 HPMC s	b	1,887	1355
bITZ + 0,25 PVP s	b	3,167	180
bITZ + 0,25 F127 s	b	1,843	1355
bITZ + 0,8 F127 s	b	4,185	1370
bITZ + 0,25 PVP sol	b	0,775	5
bITZ + 0,25 HPMC sol	b	0,774	5

T max points out the importance of nanosizing. For majority of milled samples 5-15 minutes was sufficient time to reach the peak value. Initial increase in solubility is typical for particles with increased surface-to-volume ratio. Maximal concentration (Table 9) of three milled samples was reached within 180 minutes. This time point may seem to be delayed if considering the time when the stomach is reached, but the solubility of the samples, which achieved maximal concentration in the 180th minute, fluctuates around the same solubility level and concentration at earlier time points is comparable with C max. Nevertheless, no correlation between C max and particle size of milled samples can be observed.

Solubility of bulk ITZ raises gradually and the highest value is reached after app. 24 hours. Similar pattern can be seen after creation of solid dispersion with HPMC

and F127. Solubility increase at this time point is irrelevant when considering passage of chyme through the gastrointestinal tract. Due to stronger interactions with water, both in solution added stabilisers reached C max earlier. However, their solubility curves are located below solubility curve of pure bulk ITZ.

5.4 Effect of choice of stabiliser and its concentration on solubility and on maintenance of supersaturated state

Stabiliser choice and its amount have impact on final solubility rate and the dissolved amount. As described above; F127, PVP and HPMC were utilised in this study. Especially PVP and HPMC have been reported to play an important role in maintenance of supersaturated state of ITZ (D. a Miller et al., 2008). For this reason, the milled samples were divided into following 2 groups: samples containing HPMC and those stabilised with PVP, finally the summarising table is provided (Table 10). These groups were evaluated separately. The stabilisers were added before milling in solution. Solubility curve of bulk ITZ is depicted in all the figures to provide a base line.

Solubility curves of samples milled with combination of F127 and HMPC can be seen in Figure 10. The amount of HMPC in all samples varies from 10 to 25 %. The amount of F127 differs more remarkably, from 10 % through 55 % up to 70 %. This aspect seems to be determining concentration of dissolved drug. The samples containing higher amount of poloxamer achieved superior solubility, namely sample 0,6 F127: 0,2 HPMC; 0,7 F127: 0,1 HPMC and 0,55 F127: 0,25 HPMC. Lower concentrations of F127 are apparently not as beneficial. Solubility curves of samples stabilised with F127 in 10 % and 20 % are located in lower part of graph, mentioned samples don't exceed the concentration of 2 μg/ml.

Figure 10, depicts solubility curves of samples stabilised with HPMC as a single stabiliser. These samples exhibit the highest concentration of dissolved ITZ. The amount of HPMC seems to influence initial solubility the most importantly. If HPMC in 25 % concentration is utilised, the solubility rises and a peak can be observed at the first minutes of solubility testing. Subsequently, the concentration of dissolved ITZ decreases. In 120 minutes time, concentration is only slightly higher comparing

to sample 0,1 HPMC. In both samples, concentration of dissolved drug decreases up to final measurement, unlike in the majority of solubility curves. Nevertheless, the final concentrations are high: 0,25 HPMC (1440 min) – 5,088 µg/ml; 0,1 HPMC (1440 min) – 4,544 µg/ml.

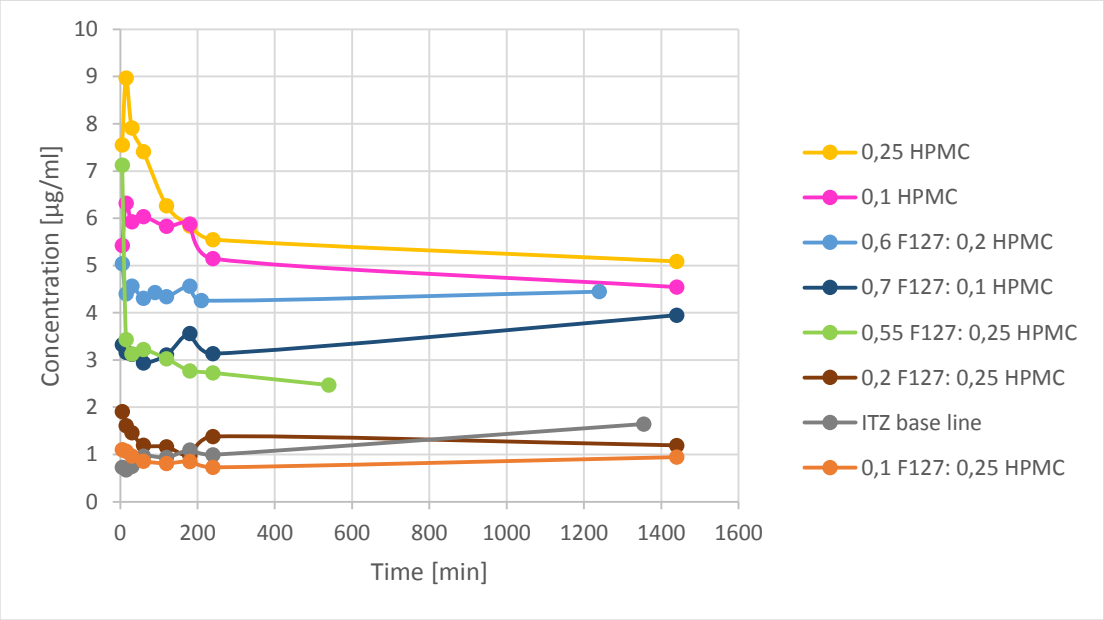


Figure 10. Solubility curves of samples containing HPMC

Following graph, Figure 11, includes the solubility of samples milled with combination of F127 and PVP or with only PVP. The content of PVP, utilised in combination with F127, can be found between 10 and 30 %, the amount of added poloxamer varies from 10 % to 70 %. Based on ITZ concentration corresponding with the amount of F127, solubility curves can be divided into two groups. Generally, the higher the content of F127 in sample, the higher the solubility obtained. This applies mainly to the first group with superior solubility results composed of F127 in 50 %, 60 % and 70 % of drug content. These compositions are able to maintain the supersaturated state as well. As the F127 amount lowers (10 % and 20 %), the solubility and ability to maintain supersaturation decreases.

Solubility of samples milled with only PVP exceeds solubility of samples where PVP and F127 were combined. Generally, higher concentration of dissolved drug is reached in samples with single HPMC compared to samples milled with single PVP. Regarding PVP samples, it is noteworthy that the higher amount

of stabiliser (40 %) does not match superior solubility. On contrary, the peak solubility is reached with 25 % of PVP, but the peak value is not maintained and the solubility decreases.

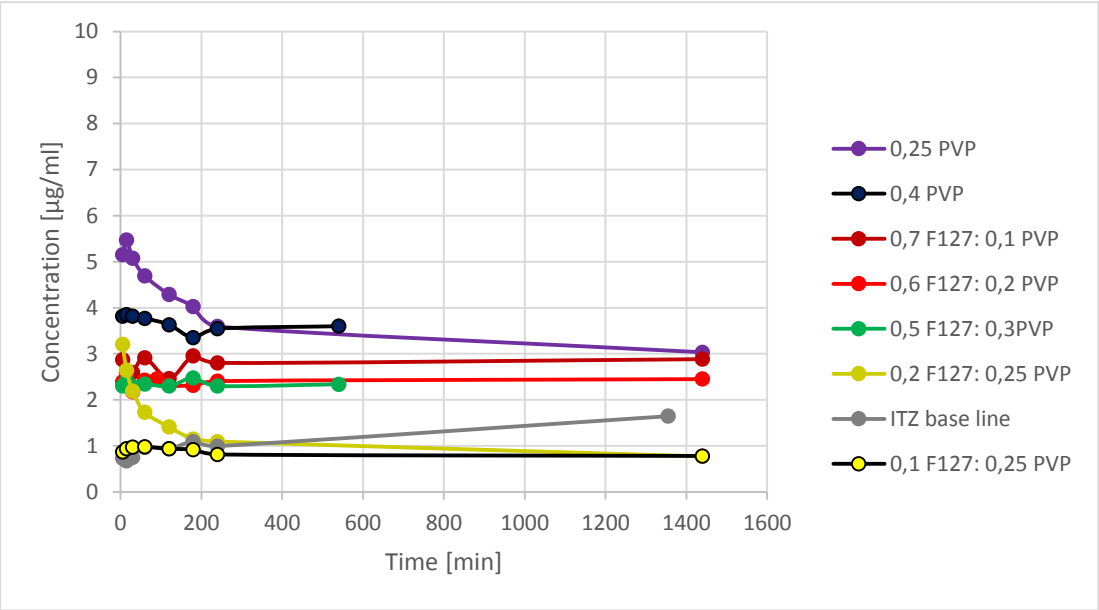


Figure 11. Solubility curves of samples containing PVP

Table 10 summarises the results obtained after performing the solubility testing. Different colours were used to mark the same stabiliser/combination of stabilisers. Close position of samples containing the same stabilisers confirm the core importance of stabiliser choice on drug solubility.

The level of solubility plays an important role over a period of the passage through the gastrointestinal tract until the absorption area is reached. Solubility of ITZ is highly pH dependent, absorption in the stomach is preferable. Therefore, concentration of dissolved drug inside 120 minutes is considered as crucial and to evaluate persistence of solubility to that time point, attention was paid mainly to period from 60 to 180 minute. Particular values of achieved concentrations of dissolved drug are provided in Table 10.

Table 10. Numerical values of concentrations of dissolved drug, aimed at 60 to 180 minute. The same stabiliser´s group are marked in the same colour.
conc. - concentration
not anal. – not analysed

Sample	initial conc. 5 min (µg/ml)	conc. 60 min (µg/ml)	conc. 120 min. (µg/ml)	conc. 180 min (µg/ml)	final conc. app. 24 h (µg/ml)
0,25 HPMC	7,554	7,411	6,268	5,838	5,088
0,1 HPMC	5,427	6,038	5,830	5,876	4,544
0,6 F127: 0,2 HPMC	5,042	4,308	4,341	4,564	4,447
0,25 PVP	5,148	4,692	4,289	4,025	3,033
0,4 PVP	3,816	3,770	3,632	3,349	not anal.
0,7 F127: 0,1 HPMC	3,322	2,940	3,110	3,561	3,949
0,7 F127: 0,1 PVP	2,873	2,912	2,464	2,953	2,883
0,55 F127: 0,25 HPMC	7,123	3,221	3,027	2,771	not anal.
0,6 F127: 0,2 PVP	2,394	2,425	2,316	2,317	2,451
0,5 F127: 0,3 PVP	2,304	2,341	2,299	2,477	not anal.
0,2 F127: 0,25 PVP	3,204	1,730	1,408	1,149	0,771
0,2 F127: 0,25 HPMC	1,907	1,198	1,161	0,983	1,194
0,1 F127: 0,25 PVP	0,872	0,983	0,939	0,919	0,781
0,1 F127: 0,25 HPMC	1,100	0,858	0,814	0,851	0,944
ITZ base line	1,646	0,968	0,939	1,094	1,646

Figure 12 displays seven samples that maintain supersaturated state, two samples that maintain their solubility level and solubility curve of bulk ITZ that provides a base line. The samples maintaining the supersaturation achieved the desired parachute effect of properly stabilised nanoparticles. 0,1 HPMC creates a plateau beginning 30 minutes after start of solubility testing. This state is maintained up to 180 minute, then the solubility decreases gradually and concentration after app. 24 hours is the lowest of the sample. Both samples 0,7 F127: 0,1 HPMC and 0,7 F127: 0,1 PVP exhibit considerable fluctuation but the overall solubility level is maintained. 0,6 F127: 0,2 HPMC; 0,6 F127: 0,2 PVP; 0,5 F127: 0,3 PVP and 0,4 PVP values do not fluctuate to that extent. Unlike 0,1 HPMC, the final concentration (after 24 hours) of the other samples raises (0,4 PVP not analysed after 24 hours) . This final increase in solubility might reflect co-solvency effect of F127.

0,1 F127:0,25 HPMC and 0,1 F127:0,25 PVP do not increase solubility very well, due to close position of their solubility curves to ITZ solubility curve it cannot be explicitly decided whether the supersaturated state is reached. However, these compositions maintain their solubility level.

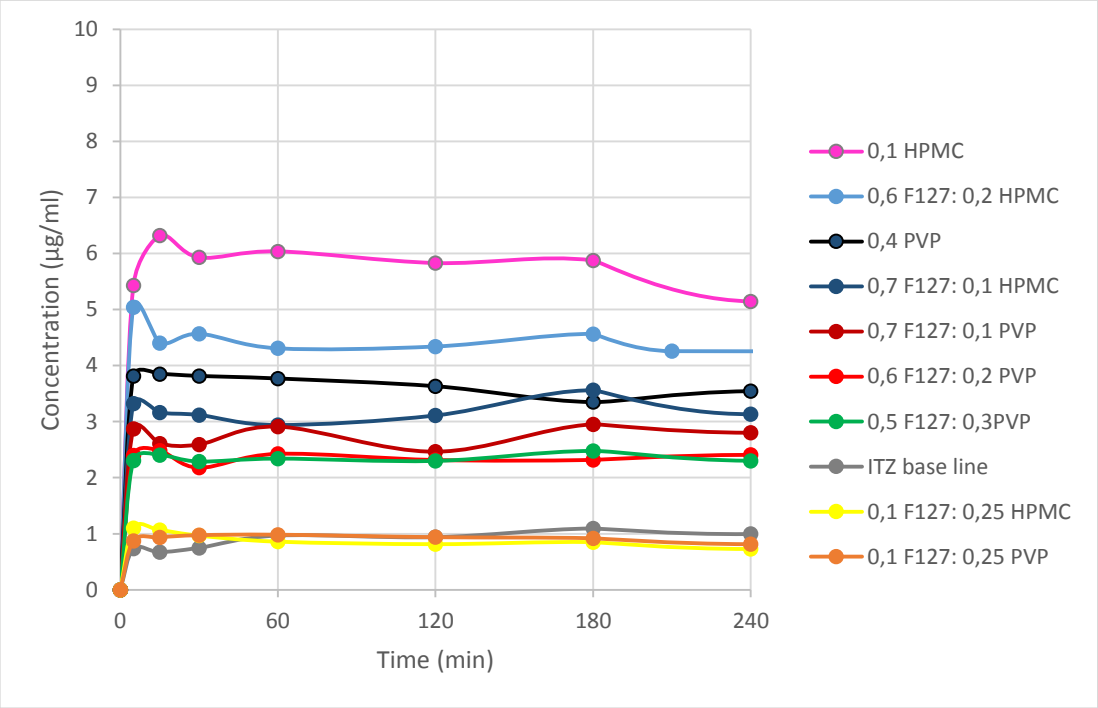


Figure 12. Summary of samples that maintain supersaturation or solubility level, aimed at 60 – 180 min. Solubility curve of bulk ITZ is depicted to provide a base line.

In Figure 13 are shown samples that reached the maximal solubility value at the very beginning and such high solubility was maintained only for a limited period of time. A high initial peak is followed by a decrease in concentration of the dissolved drug. 0,25 HPMC creates a significant peak in 15 min but then the concentration of the dissolved drug decreases and in 120 minute the concentration is the same as in case of 0,1 HPMC that creates plateau around this time point. Solubility curve of 0,25 PVP decreases gradually up to final concentration, compared to rapid drop in solubility of 0,55 F127: 0,25 HPMC that maintains approximately the same level of solubility around 120 minute but afterwards decreases as well. The initial solubility of 0,2F127:0,25 PVP and 0,2 F127: 0,25 HPMC reached in 5 minutes gradually decreases up to 180 minute, in 120 minute the concentration values are close to concentration

of dissolved bulk ITZ. It is noteworthy that 0,2 F127: 0,25 PVP continues to decrease but an increase in solubility curve of 0,2 F127:0,25 HPMC is to be found.

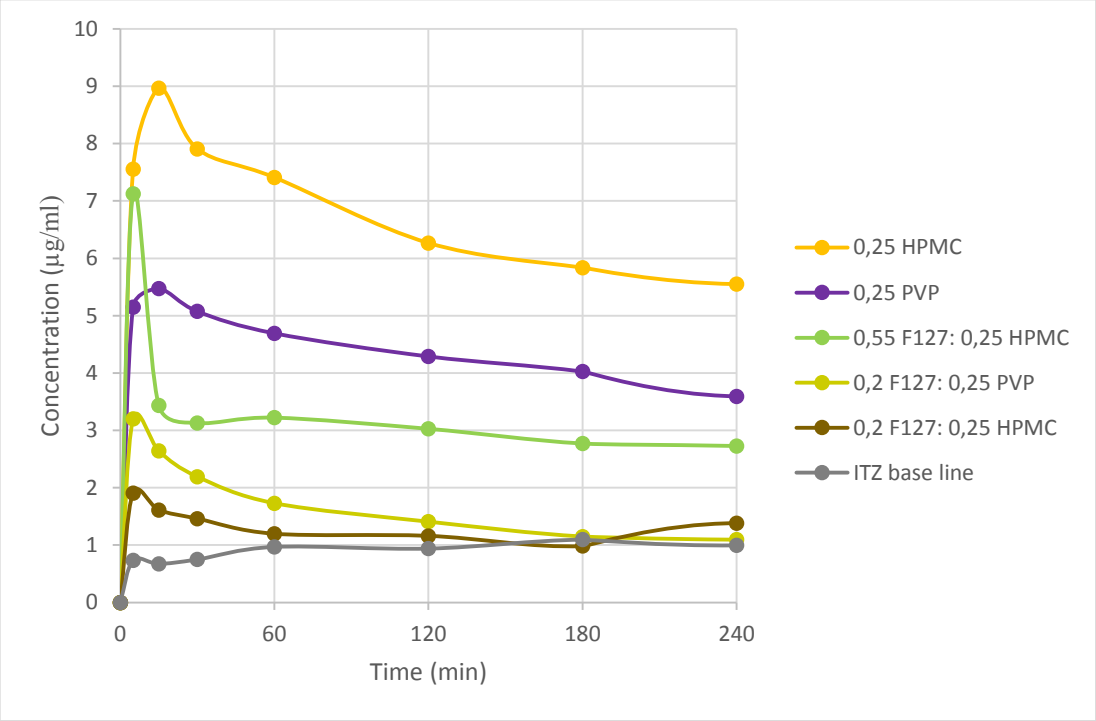


Figure 13. Summary of samples with insufficient maintenance of supersaturation, aimed at 60 – 180 min. Solubility curve of bulk ITZ is depicted to provide a base line.

Stabilisers are attached to the nanosized drug mainly by means of non-covalent intermolecular interactions, such as hydrophilic/hydrophobic interactions and hydrogen bonding. ITZ is a lipophilic weak base that acts as proton acceptor under acidic conditions. During the production phase, pH of drug suspension medium is influenced only by added stabilisers that do not generate environment acidic enough to protonate ITZ. Thus it is not charged and the ability to create hydrogen bonds is limited. Uncharged ITZ emphasizes its lipophilic properties that govern its behaviour in aqueous medium (Peeters et al., 2002)

Chemical structure of stabilisers has impact on their stabilising function. F127 acts by means of hydrophobic interactions with the drug and also employs its superior steric hindrance provided by hydrophilic tails. The length of these tails determinates the distance between newly formed particles. The mechanism of stabilisation of PVP

are hydrophobic interactions as well. Compared to F127, hydrophilic part consists of pyrrolidones that do not occupy as much space as hydrophilic tails of F127. Therefore, the thickness of steric barrier varies and results in different ability to stabilise the nanoparticles.

HPMC is an amphiphilic compound that is capable to form hydrogen bonds as hydrogen donor. It attaches with its hydrophobic parts to ITZ non-polar carbon chain, polar hydroxyl groups provide proton to form hydrogen bonds with proton acceptors, nitrogen and oxygen, within the ITZ structure. HPMC tends to attach to more hydrophilic parts of ITZ molecule. In this way it generates a proton-rich environment (D. a Miller et al., 2008) that enables creation of hydrogen bonds that play a major role in HPMC mechanism of action. Hydrophobic parts of HPMC chain interact via hydrophobic interactions with carbon chain of ITZ. These hydrophobic parts are shorter, compared to PVP or F127, thus, less efficient in interacting. Both non-covalent interactions fix HPMC tighter to ITZ molecule and enable to stay in intimate contact with drug molecule in aqueous medium. Results of solubility testing, where samples milled only with HPMC preformed the highest concentrations of ITZ, suggest positive effect of such close contact. It ensures that the nanosized drug is covered by stabiliser, however, the layer of stabiliser does not protect the nanoparticles from agglomeration. This might be reason for the biggest particle sizes contrasting with solubility test results. After inserting the agglomerates into acidic aqueous medium, their disintegration into nanocrystals leads to fast dissolution. In addition, in the acidic environment ITZ is protonated that enhances hydrogen bonding to HPMC and enables to both molecules to stay in close contact. Due to stronger interactions provided by hydrogen bonding, HPMC can inhibit precipitation more efficient and maintain supersaturated state.

From comparison of solubility curves 0,1HPMC and 0,25HPMC, the higher amount of stabiliser seems to form a better protection against agglomeration, particles are smaller and therefore the disintegration to nanoparticles is more efficient and enables to reach a significant peak in ITZ concentration.

Owing to the stabilisers mechanism of action based on attachment to the drug surface, the mixture of stabilisers has a negative impact on solubility. The stabilisers compete for place on ITZ surface to be attached. Solubility tests revealed

that excess of F127 leads to superior solubility probably due to its action as surfactant that increases also the solubility of bulk ITZ (*Figure 15*). When comparing samples combining the same amount of HPMC or PVP with F127 (for example values in 60 min: 4,307 µg/ml in 0,6F127: 0,2HPMC compared to 2,425 µg/ml in 0,6F127:0,2PVP,), higher concentrations are reached in samples with HPMC, which confirms its superior stabilising effect. The mixtures which contain lower amount of F127 than HPMC/PVP show the lowest concentrations of dissolved drug. 10 % and 20% of F127 seems to be unsufficient to solubilise and also disturbs the stabilisation provided by HPMC/PVP.

5.5 Effect of physical state (predissolved/solid) of stabiliser on solubility and maintenance of supersaturated state

Previous studies carried out with amorphous drugs have revealed the influence of addition stage and physical state (predissolved or solid) of stabiliser on its final stabilising effect (Surwase et al., 2015). Mentioned parameters were tested in this study as well.

For this purpose, two samples were milled only with F127 to prevent aggregation and obtain appropriate particle size. In order to decrease the overall amount of stabilisers, 10% and 20% of F127 were used. 10 % of F127 does not provide sufficient stabilisation of particle size, thus, only the latter sample was employed in further investigation. The sample was divided into four subsamples, HPMC or PVP was added in 25% of drug content. HPMC/PVP was predissolved in buffer solution or solid dispersion was created before addition of aqueous medium.

Figure 14 shows solubility curves of previously described experiment. It reveals a significant increase in solubility when adding PVP in a solid state (0,2F127: 0,25PVPs). This procedure does not provide sufficient maintenance of supersaturation, however, the decreasing solubility still raises above solubility curves of other formulations. In comparison, stabilising effect of HPMC is not so dependent on physical state, nevertheless, better solubility is reached with predissolved stabiliser if nanocrytalline drug utilized. In both HPMC samples a gradual increase in solubility

was present. After reaching the peak value, solubility slightly decreases, however, stabilisation of supersaturated state follows.

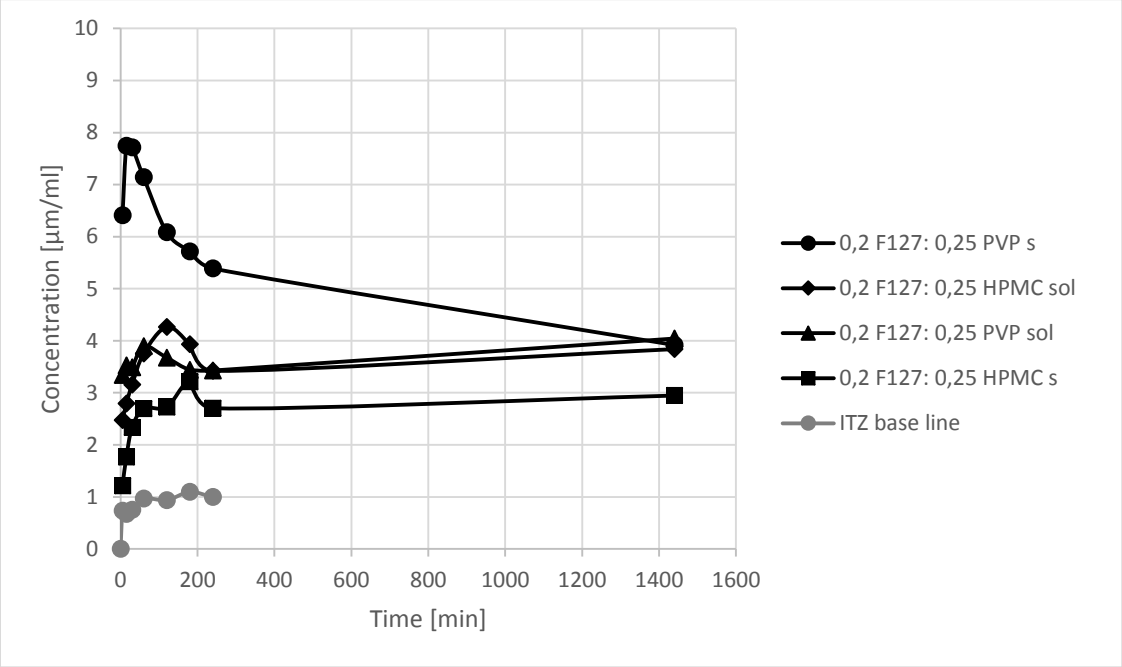


Figure 14. Effect of addition of second stabiliser to nanosized drug as solid dispersion (PVP s, HPMC s) or predissolved in buffer (PVP sol, HPMC sol). Solubility curve of bulk ITZ is depicted to provide a base line.

The influence of physical state (predissolved or solid) of stabiliser confirms also the test performed with bulk ITZ, shown in *Figure 15*. The bulk material was combined with a single stabiliser in amount 25% of drug amount, both as a solid and predissolved material. In accordance with previous results stated in Figure 14 , PVP creating solid dispersion with the drug dominates the solubility status. On contrary, the addition of HPMC as a solid stabilizer of bulk ITZ is shown more beneficial in comparison to predissolution in buffer.

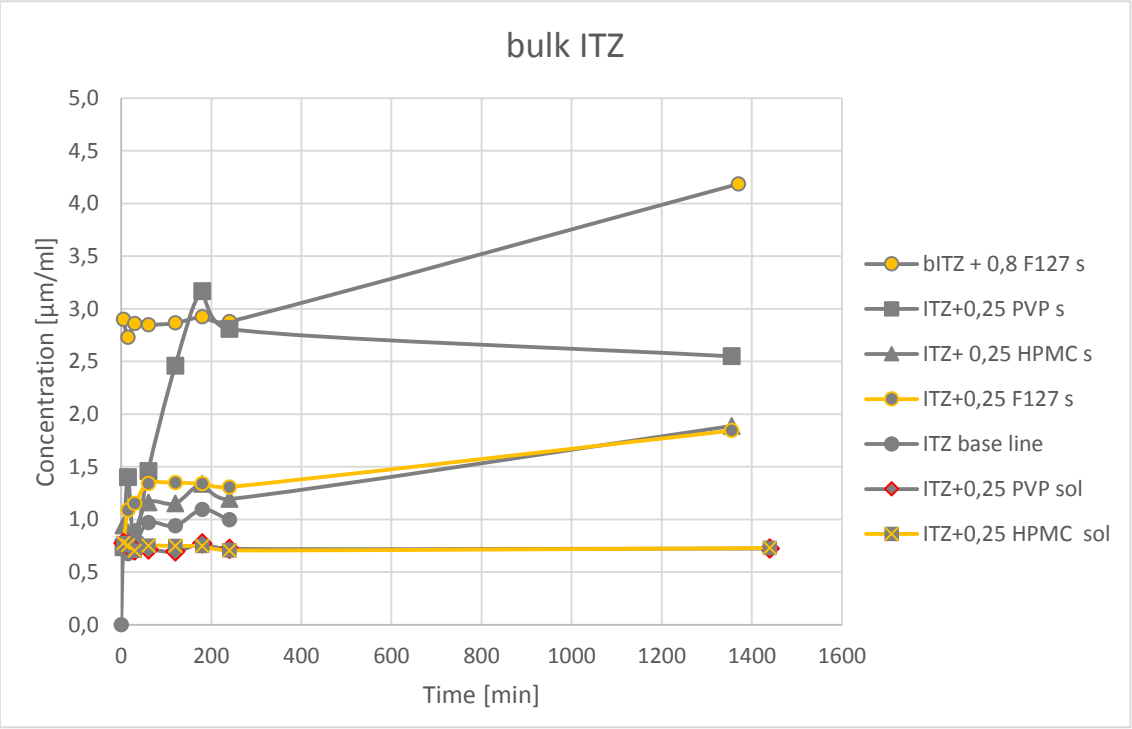


Figure 15: Effect of addition of stabiliser to bulk drug as solid dispersion (PVPs, HPMCs, F127 s) or predissolved in buffer (PVP sol, HPMC sol) to bulk drug. Solubility curves of ITZ+0,25 PVP sol and ITZ+0,25 HPMC sol overlap.

These measurements present notable co-solvency of bulk material provided by solid PVP. The ability of PVP to complex proton seems to be a key issue influencing its acting. If PVP is predissolved in acidic medium, it binds free protons and subsequently this stabiliser is less willing to interact with ITZ. From Figure 14 and *Figure 15* we can assume, that the increase in equilibrium solubility contributes to the maintenance of supersaturated state provided by PVP and also that this manner of stabilisation is more expressed than in case of HPMC. As can be seen in previous graph, the co-solvency provided by HPMC is not as efficient. This fact emphasises the inhibitory effect of HPMC on precipitation and its ability to stabilise wider gradient between equilibrium solubility and supersaturated state. F127 added as solid powder acts as surfactant and influences solubility level via decrease of surface tension that leads to enhanced wetting of drug.

5.6 Effect of addition stage of stabiliser on maintenance of supersaturated state

The second stabiliser, the stabiliser with positive impact on maintenance of supersaturated state, can be added predissolved in water before the milling (0,2F127:0,25PVP; 0,2F127:0,25HPMC) or predissolved in solubility testing medium before the test itself (0,7F127: 0,25HPMC sol, 0,7F127: 0,25PVP sol). The use of filters with various pore size limits the comparison of reached solubility levels of aforementioned samples. However, the shape and thus the ability to maintain the level of solubility is independent on filter pore size.

From Figure 16 the different tendency of the curves can be seen. Gradual increase characterises samples with HPMC/PVP added before solubility tests - 0,7F127: 0,25HPMC sol and 0,7F127: 0,2 PVP sol, their peak values are reached in 120 or 60 min, respectively. After maxima, the solubility decreases, but it is still higher or the same than the corresponding value of bulk itraconazole.

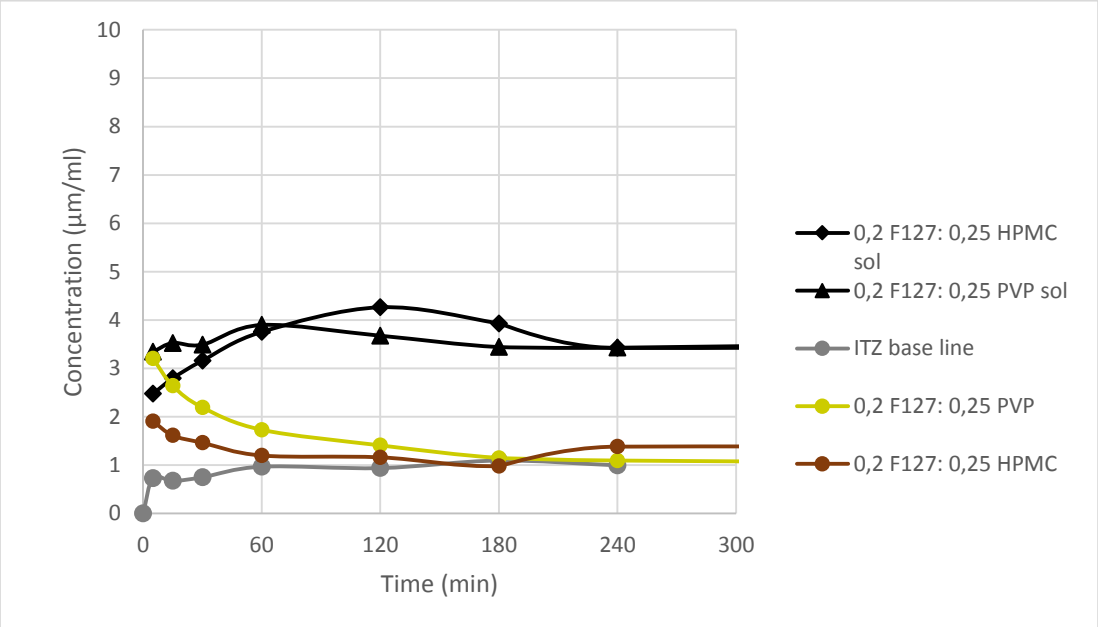


Figure 16: Solubility curves of samples of the same composition but various addition stage of the predissolved second stabiliser – before milling (0,2 F127:0,25 PVP; 0,2 F127:0,25 HPMC) or before solubility testing (0,7 F127: 0,25 HPMC sol, 0,7 F127: 0,25 PVP sol, aimed at 60 – 120 min. Solubility curve of bulk ITZ is depicted to provide a base line.

This missing “parachute effect” when both the stabilizers are added before the milling might be explained by insufficient access of HPMC/PVP to drug molecule when dissolved. As the nanosized crystalline ITZ is milled, a layer of F127 covers it and hinders its contact with the other stabiliser. Intimate contact between the drug and the polymer precipitation inhibitor (HPMC/PVP) seems to be crucial in maintenance of supersaturation (D. a Miller et al., 2008). General increasing tendency of the curves could be attributed to co-solvency effect of all the stabilisers. On contrary, the mixture of stabilisers added in solution before the milling - 0,2 F127:0,25 PVP and 0,2 F127:0,25 HPMC - seems to be insufficient to maintain the supersaturated state reached in first minutes of the test. Stabilisers seem to compete for binding place on ITZ particles surface, their interaction leads to disturbance in their stabilising function.

6 Conclusions

Fifteen different compositions of itraconazole (ITZ) nanocrystals were prepared by wet milling. Fixed combinations of stabilisers (F127+HPMC and F127+PVP) in various ratios or single stabilisers (HPMC and PVP) were applied to analyse their impact on solubility and maintenance of the supersaturated state.

The solubility tests have revealed that the importance of nanosizing lies in the time, when the maximal concentration is reached (T max) and in the level of maximal concentration as well. Nanoparticles enable to reach maximal concentration of dissolved drug when in contact with absorption area of the gastrointestinal tract. Nanoscale was achieved in all samples except two milled with HPMC only. Possible agglomeration of nanoparticles stabilised by HPMC may explain exceeding 1000 nm particle size. Nevertheless, no correlation between T max, solubility or maintenance of supersaturation and particle size of milled samples was observed.

The obtained data indicated that the choice of stabiliser influenced solubility value and persistence of its level the most importantly. Single stabilisers reached higher ITZ solubility than most of combinations, the order was concluded as followed: HPMC>PVP>F127+HPMC>F127+PVP. In case of combining, higher amount of F127 was beneficial. Effect of stabilisers is supposedly based on intermolecular interactions, hydrogen bonds have a stronger impact than hydrophobic interactions. Around 120 min absorption time point the solubility was from two to six-fold as compared to the solubility of bulk drug under the same conditions, depending on the sample composition. The supersaturation was maintained with various degree of success, positive contribution of higher F127+HPMC/PVP ratio can be concluded.

The results proved that physical state (predissolved/solid) of stabiliser influences solubility and maintenance of the supersaturated state as well. This aspect is stressed in case of PVP, which as a hygroscopic polymer interacts strongly with water molecules if predissolved. Thus its addition as solid dispersion reaches higher solubility values. On the contrary, superior maintenance of supersaturation is provided by HPMC and predissolved PVP.

The stage at which the stabilisers are added determinates their interactions on surface of poorly soluble drug. Measurements suggest that milling with single F127

that hinders particle aggregation and addition of precipitation inhibitor (HPMC/PVP) after milling have positive impact on maintenance of solubility level.

In conclusion, 0,1 HPMC reaches the highest maintained solubility level and forms a beneficial plateau up to 180 minute of the test. 0,25 HPMC achieves the highest solubility level; no plateau is formed but the concentration raises over concentration values of 0,1 HPMC. 0,4 PVP provides sufficient stabilisation of its solubility, as well. Also samples that combine 0,7 F127 and 0,6 F127 with HPMC (10% and 20% respectively) or PVP (10% and 20% respectively) achieved a considerably high level of supersaturation. Beneficial solubility values, sufficient maintenance of supersaturation and uncomplicated preparation make these sample worth further investigation.

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List of abbreviations

Acronym	Definition
BCS	biopharmaceutics classification system
BET	Brunauer, Emmett and Teller technique
C max	maximal concentration
DLS	dynamic light scattering
DSC	differential scanning calorimetry
EP	meets European Pharmacopeia requirements
F127	poloxamer 407
GHP	hydrophilic polypropylene
HPH	high-pressure homogenisation
HPLC	high-performance liquid chromatography
HPMC	hydroxypropyl methylcellulose
HPMCAS	hydroxypropyl methylcellulose acetate
ITZ	itraconazole
LV	low viscosity
PDI	polydispersity index
PEO	polyethylene oxide
PPO	polypropylene oxide
PVP	polyvinyl pyrrolidone/povidone
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
T max	time when the maximal concentration is reached
TEM	transmission electron microscopy
TPGS	D-a-tocopherol polyethylene glycol 1000 succinate
UV	ultraviolet

VT-XRD	variable temperature X-ray diffraction
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

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