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Miniaturized and fast method for solubility and level of supersaturation determinations of drug nanocrystals

Diploma Thesis

Division of Pharmaceutical Chemistry and Technology

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Hradec Králové, 2017   Romana Paľová

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ANNOUNCEMENT

I declare that this diploma thesis has been worked out by myself and that all literature and other resources, I used while processing, are listed in the literature list and are properly cited. The master thesis was not used to obtain different or the same title.

In ………………. date ………….   …………………….

Romana Paľová

Abstract

Title of thesis: Miniaturized and fast method for solubility and level of supersaturation determinations of drug nanocrystals

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As a formulation strategy for improving the bioavailability of poorly water-soluble drugs, drug nanocrystals can be employed. Several studies have shown that drug nanocrystals exhibit higher dissolution rate compared to conventional drug formulations. However, when orally administered, various conditions in gastrointestinal tract, such as changes in pH may result in problematic absorption. In this work, the preparation of drug nanosuspension of itraconazole (ITZ), as model poorly water-soluble compound, was carried out. The nanosuspension was prepared by using wet milling method of preparation, with suitable stabilizers added (Poloxamer 407 and Hydroxypropyl methylcellulose). Hydrochloric acid buffer pH 1.2 was chosen to create an environment similar to gastric fluids. In order to investigate changes in solubility of ITZ nanosuspension, UV spectrophotometer was used. Measurement was performed at the wavelength 551 nm, where dissolved ITZ does not absorb light and no change in absorbance occurred. Subsequently, the conditions similar to those, when drug reaches the main site of absorption, i.e. small intestine, were simulated by raising the pH value to 7. Results showed rapid increase in absorbance caused by precipitation of ITZ in the analyzed sample. Thus, this simple method is a promising tool to determinate solubility and level of supersaturation of drug nanocrystals.

Key words: itraconazole, drug nanocrystals, supersaturated state, precipitation,

Abstrakt

Názov diplomovej práce: Miniaturizovaná a rychlá metoda pro stanovení rozpustnosti a stupně přesycení nanokrystalů léčivých látek.

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Nanokryštály liečiv môžu byť používané ako formulácie pre zlepšenie biodostupnosti liečiv, ktoré sú vo vode zle rozpustné. Niekoľko štúdií dokazuje, že nanokryštály vykazujú lepší disolučný profil v porovnaní s bežnými formuláciami. Avšak po perorálnom podaní môžu podmienky v gastrointestinálnom trakte, ako napríklad zmeny v pH, spôsobiť problémy so vstrebaním liečiva. V tejto práci bola vlhkým mletím pripravená nanosuspenzia itrakonazolu, ako predstaviteľa vo vode zle rozpustnej zlúčeniny. Pridané boli vhodné stabilizéry (Poloxamer 407 a Hydroxypropylmetylcelulóza). Pufr s pH hodnotou 1.2 bol vybraný pre vytvorenie prostredia podobného tomu v žalúdku. Zmeny v rozpustnosti boli sledované pomocou UV spektrofotometra, pri nastavení vlnovej dĺžky 551 nm, kde rozpustený itrakonazol neabsorbuje svetlo. Zmeny v absorbancii neboli zaznamenané. Následne zvýšením pH na hodnotu 7 boli simulované podmienky ktoré nastávajú keď liečivo prechádza zo žalúdku do tenkého čreva - hlavného miesta absorpcie. Výsledky ukázali rapídny nárast v absorbancii, spôsobený vyzrážaním itrakonazolu v analyzovanom vzorku. Z toho vyplýva, že popísaná jednoduchá metóda má potenciál čo sa týka určovania rozpustnosti a stupňa presýtenia nanokryštálov.

Kľúčové slová: itrakonazol, nanokryštály, presýtenie, zrážanie,

List of abbreviations

ITZ	Itraconazole
BCS	Biopharmaceutics Classification System
API	Active Pharmaceutical Ingredient
BAV	Bioavailability
DLVO	Derjaguin, Landau, Vervey, and Overbeek
DLS	Dynamic Light Scattering
PCS	Photon Correlation Spectroscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
GIT	Gastrointestinal tract
DSC	Differetial Scanning Calorimetry
XRPD	X-ray Powder Diffraction
NCS	Nanotoxicological classification system
HPMC	Hydroxypropyl methylcellulose
F 127	Poloxamer 407
UV	Ultraviolet
PPO	Poly(prolynene oxide)
PDI	Polydispersity Index
LD	Laser Diffraction
PVP	Polyvinylpyrrolidone
USP	United States Pharmacopeia
PEO	Poly(ethylene oxide)

Contents

1. Aims

Solubility of poorly soluble drugs are often enhanced by utilizing their metastable forms. However, the problem with these metastable forms are that they may precipitate if the dissolution environment is altered, e.g. in GI tract. In order to study the stability of these metastable forms, like drug nanocrystals, fast and small scale solubility testing system is needed. Accordingly, the aim of the study was to develop miniaturized and fast method for reliable solubility and level of supersaturation determinations of drug nanocrystals.

2. Theoretical section

2.1 Poorly water soluble drugs

New chemical entities are required to have an acceptable bioavailability in order to be efficient enough. The inadequate saturation solubility and the consequently limited dissolution rate usually result in low concentration gradient between the gut and blood vessel which may lead to poor oral bioavailability and/or erratic absorption. Thereby, the aqueous solubility is fundamental property which should be taken into account in order to efficient absorption after oral administration. The poor oral bioavailability of orally administered drug is usually due to low aqueous solubility. Because of solubility problem of many drugs the bioavailability of them gets affected, thereby solubility improvement becomes necessary. In addition to greater bioavailability, smaller doses can be used and hence the *in vivo* toxicity is reduced.

The solubility of a solute can be defined as the maximum quantity of solute that can be dissolved in a certain quantity of solvent or quantity of solution at a specified temperature. Currently, approximately 40% of the marketed immediate release oral drugs are regarded as practically insoluble (<100 µg/mL) (Takagi et al., 2006).

Thereby enhancement of water solubility remains one of the most challenging tasks of drug development.

Solubility exists at dynamic equilibrium, resulting from two simultaneous reactions. Usually between dissolution process and phase joining (e.g. precipitation), and the rates of the forward and reverse reactions are the same time. Under certain conditions, solubility may be exceeded resulting in a supersaturated solution, which presents, however, metastable state.

There are many factors which can influence the solubility. The first of them - the particle size is also one of the factors manipulated in this work mentioned below. Temperature is found as the other determinant of solubility because solubility will increase (in the most cases) as the system receives energy in the form of temperature. The other factor - pressure have a considerable influence mainly in gas solutions.

Our model compound - itraconazole is a representant of The Biopharmaceutics Classification System (BCS) - class II. Which means it is poorly soluble but permeable. So, in this case enhancement of solubility leads to enhancement of bioavailability. BCS

serves as a scientific classification that takes into account solubility and intestinal permeability which both govern the rate and extent of bioavailability after oral administration. (Amidon, G. L., et al., 1995)

2.1.1 Itraconazole

ITZ is a triazole antifungal drug with a broad-spectrum activity against the number of fungal species, as well as a favorable pharmacokinetic profile. The mechanism of action is based on its binding of fungal cytochrome P-450 isozymes with inhibition of ergosterol synthesis and malfunction of the cell membrane as a result.

Itraconazole was used in this experiment as a model compound and a representant of the BCS II. Class (poor water-soluble, permeable).

Pharmacokinetic profile determines the time course of drug concentrations in various parts of the body for any given dose. Simultaneously, this profile affects the clinical efficacy of the drug (Böhme, A., et al., 1996).
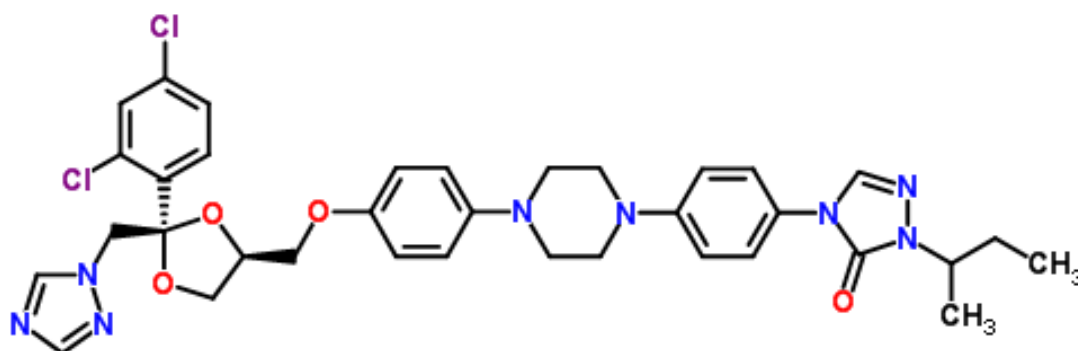

Figure 1. Chemical structure of itraconazole (R 51 211), (+-)-cis-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(lH- 1,2,4-triazol- 1-ylmethyl)- 1,3-dioxolan-4-yl] methoxy]phenyl] - 1 - piperazinyl]phenyl] - 2,4 - dihydro - 2 - (1 - methylpropyl)-3H-1,2,4-triazol-3-one

Itraconazole has three nitrogen atoms in its azole ring which can result in improved tissue penetration, prolong half-life as well as increase specificity for fungal enzymes (Hardin, T. C., et al., 1988).

The pK_a of itraconazole is 3.7 what makes it an extremely weak base, which is ionized at a low pH. Itraconazole is insoluble in water due to its high lipophilicity (Prentice, A.G., et al., 2005). The solubility of weakly basic compounds tends to be significantly higher in the ionized form (for instance presented in the stomach environment) than in the unionized form (in the small intestine).

When administered orally, peak plasma concentration is reached within 1.5 to 4 hours (a difference in the extent of its absorption among individuals must be taken into account). The bioavailability of conventional ITZ is relatively low after oral administration, especially when given on an empty stomach, thus, it is recommended to take ITZ with a meal in order to maximize its therapeutic effect.

The tissue penetration of ITZ is high and sustained. Besides its wide distribution in the body, higher levels of concentration (up to 10 times higher than corresponding plasma concentrations) can be found in some tissues. On the other hand, low to negligible concentrations are achieved in cerebrospinal fluid (Grant, S.M., et al., 1989).

2.1.2   Formulation options for poorly soluble drugs

Aiming at addressing the problems, several strategies have been employed in order to help to improve solubility. These include physicochemical modifications and some other techniques. The techniques involve decreasing particle size (micronization or nanonization), formation of polymorphs/pseudopolymorphs (including solvates), amorphous state, preparation of drug dispersion in carriers such as solid dispersion, eutectic mixtures, synthesis of soluble prodrug, salts formation, change of pH and complexation, the addition of adjuvants such as surfactants and novel excipients, solubilizers, cosolvency, supercritical fluid process, etc. (Ketan T. S., et al., 2013). Additional approaches comprising vesicular systems like dispersion of solids, liposomes, emulsion and microemulsion methods, and inclusion complexes with cyclodextrins, can be employed, but unfortunately, the major issue of all these methods is lack of universal

applicability to all drug candidates (in regard to their certain chemistry, molecular size or conformation (Agrawal, Y. K. et al., 2011).

2.2 Nanocrystals

Drug nanocrystals can be described as crystals with a size at nanoscale, which means they are nanoparticles with crystalline character.

Recently, nanotechnology has become one of the most promising, effective approaches to increase the dissolution rate of drug and consequently bioavailability after oral administration. It is shown to be a simple, universal and industrially feasible way to improve drug solubility (Müller, R. et al., 2008).

Nanosizing is defined as a reduction of particle size down to submicron range (recent advances in milling technology achieves size range 100-500 nm). Generally, the particle size and shape play an important role in drug fabrication because they have an influence not only on bioavailability but also affect mixing, granulation, compression, and coating of solid dosage forms. Particle size reduction seems to be the easiest and powerful formulation approach of improving solubility, dissolution velocity, as well as decreasing side effects of a drug. Figure 2 depics changes when changing drug particle size from microsized to nanosized.
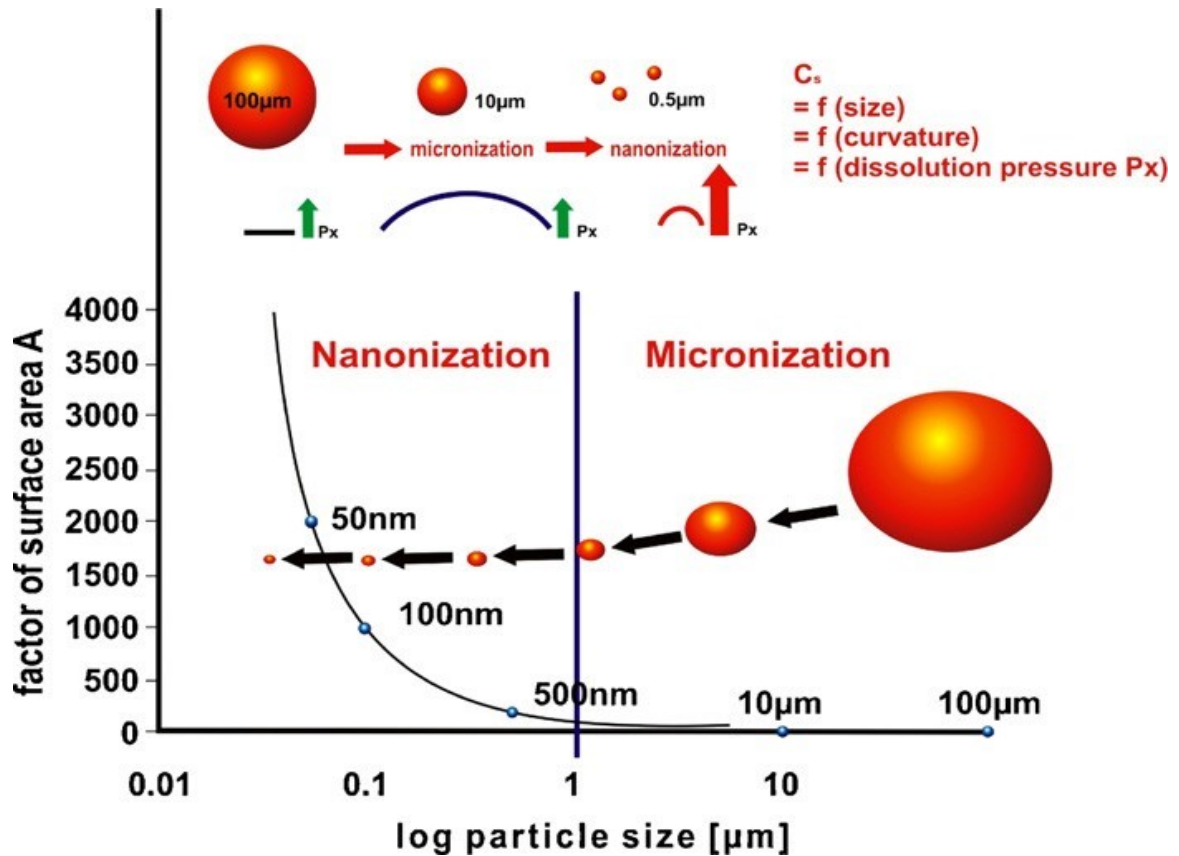
Figure 2. Comparison of microsized and nanosized particles properties: change in properties during nanosizing. Upper: the curvature increase resulting in increased dissolution pressure. Lower: the increase in surface area being pronounced below 1mm and very pronounced below 100 nm (Shegokar R et al., 2010).

Besides, drug nanocrystals possess another special feature because they can markedly increase an adhesiveness to surface or cell membranes. This can be important for those drugs not immediately dissolved in the GIT. The mucous gel layer represents a porous structure for nanoparticles. Because of the suitable size, they are capable of penetrating deeply into the gel layer and get in close contact with the mucous network. The size dependency of particle deposition has been found.

Therefore, the ability of an increased adhesiveness helps to prolong the contact time with the GIT membrane, passive diffusion is enhanced. In addition, particles captured by mucosal layer are protected from denaturation in the GIT lumen (Ponchel, G., et al.,

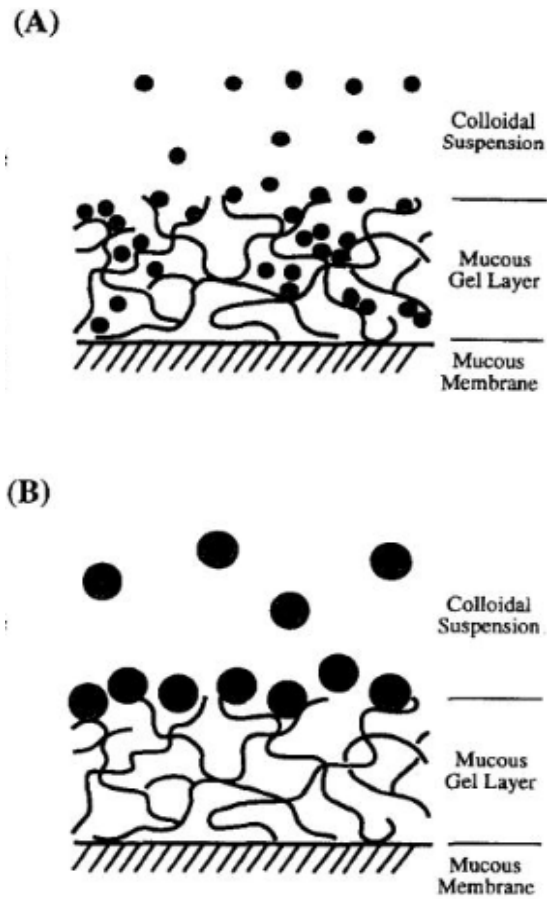1997). Figure 3 depicts the possible mechanism of increased mucoadhesiveness of drug nanocrystals.

Figure 3. Adsorption models A: in the case of nanosized particles B: in the case of microsized particles (Ponchel G., 1997)

As described in this chapter above, nanocrystal systems exhibit several advantages compared to conventional pharmaceutical formulations, briefly shown in Table 1 (Shedokar, R., et.al., 2010, Gao, L., et.al., 2008).

Table 1. Advantages of nanosuspensions over conventional formulations.

Route of administration	Disadvantages of conventional formulations	Benefits of Nanosuspensions
Oral	Slow onset of action/poor bioavailability	Rapid onset of action/improved solubility so improved bioavailability
Ocular	Lacrimal wash off/ low bioavailability	Higher bioavailability/ dose consistency
Intravenous	Poor dissolution/ non-specific action	Rapid dissolution/ tissue irritation
Intramuscular	Low patient compliance due to pain	Reduced tissue irritation
Inhalations	Low bioavailability due to low solubility	Rapid dissolution/ high bioavailability/ dose regulation

The fundamental disciplines dealing with enhancement of dissolution velocity of drug nanocrystals always take into account two following equations: Ostwald–Freundlich equation and Noyes-Whitney equation. **Ostwald–Freundlich equation** expresses the influence of particle size on saturation solubility (C_s), a compound-specific constant depending on the temperature in a certain solvent. With a decrease of particle size saturation solubility increases as a result of increased specific surface area of the phase (Equation 1):

$$log \frac{C_s}{C_a} = \frac{2\sigma V}{2.303RT\rho r}$$

Equation 1. Ostwald-Freundlich equation,

where C_s is the saturation solubility (for particle with radius r), C_a is the solubility of the solid comprising of large particles, δ is the interfacial tension of compound, V is the molar volume of the material, R is the gas constant, T is the absolute temperature, p is the density of the solid, r is the radius.

According to **Noyes-Whitney equation**, when the particle size is reduced, the total exposed surface area is increased, hence the dissolution rate is enhanced. In other words, when the particle becomes smaller, drug particles can interact more with the solvent which results in increased solubility. Additionally, the diffusion layer thickness is also reduced by this process, therefore the concentration gradient is also enhanced (Noyes, A.A., Whitney, W.R., 1897) (Equation 2).

$$\frac{dW}{dt} = \frac{kA(C_s - C)}{L}$$

Equation 2. Noyes-Whitney equation,

where dW/dt means the rate of dissolution, A is the solute surface area in contact with the solvent, C stands for the drug concentration in the bulk solvent (gastrointestinal fluids), C_s is saturation solubility of the drug within the diffusion layer , k stands for the diffusion coefficient, and L is the thickness of the diffusion layer around each drug particle (Skyner, R., et al., 2015).

As an illustration of this equation, the following schema can be depicted (Figure 4.).
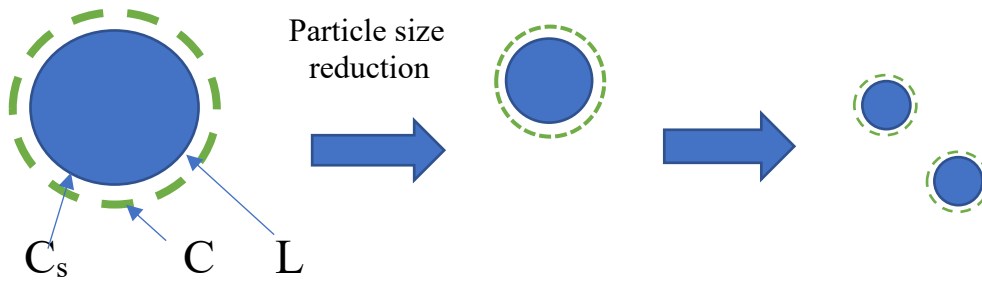
Figure 4. Noyes-Whitney equation explained by simplyfied schema.

Nanocrystals consist of 100 % Active Pharmaceutical Ingredient (high drug is typical, as the particle core consist of pure drug material), suspended in dispersion stabilized with certain excipients such as polymers or/and surfactants. Dispersion media can be water, aqueous solutions or nonaqueous media. Generally, nanocrystals can be used in order to improve the solubility of drugs that are poorly soluble in water as well as lipid media. (Müller, R. et al., 2008). In the strictest sense, such an amorphous drug nanoparticle should not be called nanocrystal. However, often one refers to "nanocrystals in the amorphous state". Table 2 can serve in order to summarize the main nanocrystals properties Table 2 can serve.

Table 2. Summary of some important drug nanocrystal properties (Müller, R., et Junghanns, 2008).

Properties of drug nanocrystals
1 Particle size below 1 μm
2 100% Drug, no carrier
3 Generally needed to be stabilized by surface active agent
4 Crystalline or amorphous structure (Amorphous state offering advantages)
5 Increase in saturation solubility
6 Increase in dissolution velocity

2.3 Preparation techniques

Very broadly, there are two ways how to produce nanoparticles. The first one starts with bulk material and then break it into smaller particles using a mechanical, chemical or another type of energy. This technique is called **„top down"** and includes pearl milling, high-pressure homogenization, as well as the combination of both technologies (Merisko-Liversidge, E., et.al., 2003). On the other hand, the second approach called **„bottom up"** starts with dissolved molecules subsequently precipitated by mixing with a non-solvent. A large number of nuclei is formed within the supersaturated drug in the antisolvent. The formed nuclei keep growing until nanocrystals are created. Thus, in this technique, nanoparticle material is built *via* chemical reactions (anti-solvent precipitation, supercritical fluid process) (Müller, R., et Junghanns, 2008, Müller, R., et.al., 2003). These two ways are described in Figure 5.
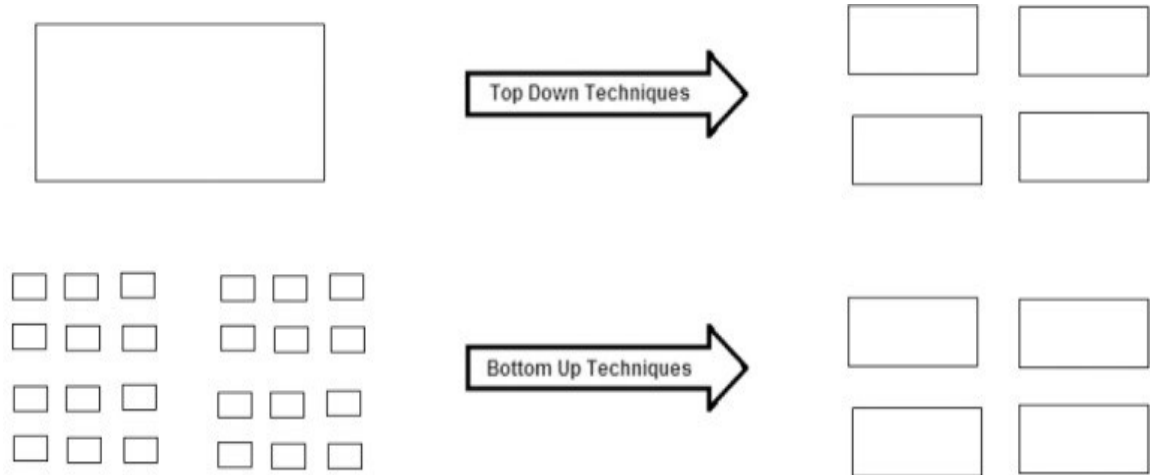
Figure 5. The main difference between Top Down and Bottom Up Techniques.

For the comparison, three different preparation techniques are briefly described in Table 3. (Shegokar R et al., 2010, Hao et al., 2012; Keck & Müller, 2006; Gao, L., et.al., 2008).

Table 3. Important advantages and disadvantage of the most important preparation techniques.).

Method	Advantages	Disadvantages
High-pressure homogenization	Ease of scale-up and little batch-to-batch variation Narrow size distribution in the final product, allowing aseptic production of nanosuspensions for parental administration and flexibility in handling the drug quantity Ease of scale up	Pretreatment of micronized drug particles Pre-suspending material before subjecting it to homogenization Energy intensive method
Milling	The same as those for high-pressure homogenization	Potential erosion of material from milling pearls Expensive equipment Intensive energy Long times Drug loss
Microprecipitation	Low need for energy Low cost Stable products Simple operation process Continuous production	Narrowly applying space Wide size distribution Potential toxicity of non - aqueous solvents Difficult to choose the solvent and antisolvent

During processing of drug microcrystals to drug nanocrystals, either crystalline or an amorphous product can be produced, the latter one especially when applying precipitation. It mainly depends on the type of used method. All the methods mentioned above result in production of solid drug nanoparticles coated with stabilizer/s.

There are many important factors which influence the success of nanocrystal formulation. The following ones are perhaps the most important: physical stability, solubility (which

affect supersaturation and dissolution) and selection of suitable excipient (Tuomela, A., et.al., 2016).

2.3.1   Wet milling technique

Wet milling is considered a standard method employed in pharmaceutical formulation development.

In general, in this grinding method, high-shear energy is generated by the movement of the milling media. This leads to particle size reduction. The milling process must be performed under certain conditions which are controlled. Various milling media usually composed of zirconium oxide , glass, or highly cross-linked polystyrene resin can be used. Media chamber is fed with milling media, water drug, as well as the stabilizer (Figure 6). The process always needs to be under controlled temperature. Milling media are rotated while the very high-shear rate is reached. The method gives a narrow size distribution product. On the other hand there are some disadvantages such as erosion of milling pearls resulting in trace residues contained in the product material. The milling time varies from 30 mins to several hours, or days, depending on the hardness of the drug, temperature, viscosity, size of milling media, energy input and surfactant concentration as well (Shegokar, R., et al., 2010). The efficienty of the milling strongly depends on the size of the milling media and the intensity of grinding energy (Ghosh, I., et al., 2011).
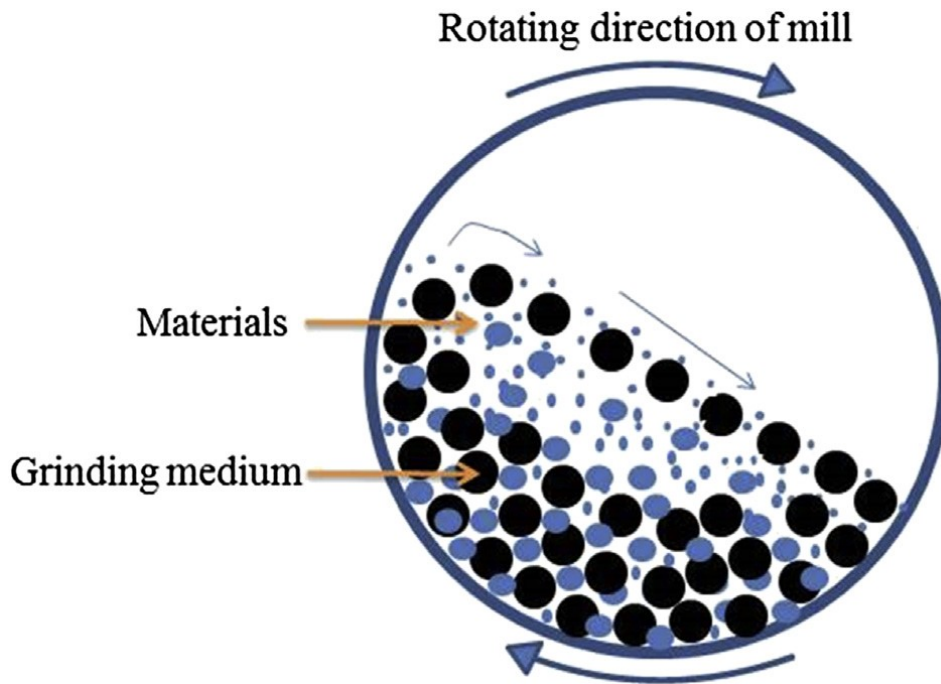
Figure 6. Schematic illustration of ball milling technique, retrieved from Khadka, P., et al., 2014.

2.4 General stability problems of nanosuspensions

There are some main physical instabilities, which negatively influence the quality of formulations and productions. These include e.g. sedimentation/creaming, agglomeration, crystal growth and change of crystalline form. For instance, the agglomeration is crucial during formulation of pulmonary administered drugs, and also of intravenous delivery because of the risk of embolization.

Besides, preparation process has an impact on the stability and robustness of nanosuspension.

2.4.1   Crystal growth

In submicron colloidal dispersion, this phenomenon is known as **Ostwald ripening**. This process is driven by the concentration gradient between small and large particles as the system tries to reduce its overall energy.

The molecules at the surface of smaller particles tend to detach and diffuse, and subsequently attach to the surface of larger particles (which is energetically more favorable). In other words, the smaller particles are more soluble than the larger ones, thus mass transfer occurs from the fine to the coarse particles.

The process of **nucleation** presents the first step in the formation of new thermodynamic phase/structure. The stage of nucleation is considered to be an activation energy barrier to the spontaneous crystallization from a solution and thus, it determines how long it takes the new structure to appear. Nucleation is considered to be very sensitive to impurities in the system.

Two types of nucleation can possibly occur: homogeneous and heterogeneous. The heterogeneous type involves a surface (such as impurity surface) of the different composition onto which the solute crystallizes. It is explained by the energy barrier decrease in the presence of impurities.

Generally, in order to avoid Ostwald ripening phenomenon, the narrow size distribution of drug nanocrystals should be concerned. This issue is more serious in polydisperse suspension but it does not play such an important role in uniform nanosized systems. Mechanical homogenization and addition of stabilizers are available to avoid this problem.

2.4.2 Agglomeration

Agglomeration automatically occurs when the particle size is at the nanoscale. Due to decreased particle size, and enlarged surface area, the free Gibbs energy is drastically increased. Thus, the nanoparticles agglomerate spontaneuosly in order to decrease the unfavorable surface energy. Stabilizer introduction presents the most effective way of preventing agglomeration by generating electrostatic and steric stabilization.

The **DLVO theory** can be applied: this theory describes the forces between charged surfaces interacting through a liquid medium. This includes the effect of Van der Waals attractions and the electrostatic repulsion due to so-called double layer surrounding counterions. Therefore, when adding suitable (charged surfactant) stabilizer, electrostatic stabilization occurs via thickening the electronic double layer as a result of adsorption of charged molecules onto the particle surface. Steric stabilization could be obtained with amphiphilic or non-ionic stabilizers absorbed onto the surface (Wang, Y., et al., 2013, Pawar, V.K., et al., 2014).

2.4.3 Sedimentation

In formulation medium, the particles have a tendency to settle down. Arising from Stokes′s principle of sedimentaion, the velocity depends on prticle size, medium viscosity and density difference between nanoparticles and medium (Equation 3).

The addition of stabilizers results in shorter density between the two phases and also the viscosity of medium is increased, thereby sedimentation is not a serious issue here.

$$\frac{dx}{dt} = \frac{d^2(\rho_1 - \rho_2)g}{18\eta}$$

Equation 3.

Dx /dt is the rate of setting, d is the diameter of the particles, p_1 is the density of the particle while p_2 is the density of the medium, g is gravitational constant and n is the viscosity of the medium

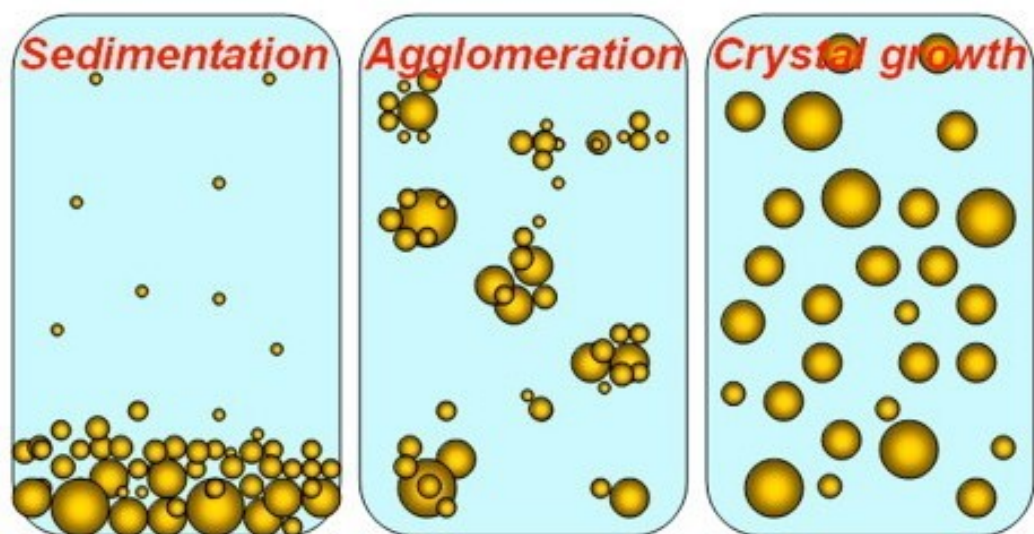For the record, stability issues in nanosuspensions are illustrated in the following Figure 7:

Figure 7. Stability problems of nanosuspensions. Retrieved from Wu, L., et al., 2011.

2.5 Characteristics of drug nanocrystals

2.5.1 Morphology, particle size, and distribution

Particle size is the crucial determinant of saturation solubility and thus dissolution rate. It is known that narrow size distribution prevents agglomeration and Ostwald ripening.

There are some devices providing analysis of these properties. These analyses are listed in the following Table 4 (Peltonen, L., et al., 2015; Gao, L., et al., 2008).

Table 4. Various methods of drug nanoparticles characteristics analysis.

Method	Information about/capability of:
DLS (Dynamic Light Scattering) PCS (Photon Correlation Spectroscopy)	particle size, particle size area, particle size distribution such e.g. polydispersity index,
LD (Laser Diffraction)	detecting agglomerations
SEM (Scanning Electron Microscopy)	Regristration of morphology changes, particle size, and shape analysis
TEM (Transmission Electron Microscopy)	Investigating density, particle size and shape analysis.

2.5.2　Zeta potential

Zeta potential (particle surface charge) is determined from the mobility of particle under applied electric zone. This property gives certain information about the long-term physical stability of the nanosuspension. Both stabilizer and drug itself govern the zeta potential. It is generally determined that zeta potential value higher than 60 mV signs extreme stability, on the other hand, if the stabilization is only based on charge, values lower than 5mV will induce fast aggregation of particles. However, in the case of stabilization with only electrostatic repulsion, the absolute value is required to be at least 30 mV (Hanaor, D., et al., 2012; O´Brien, R.W., et al., 1990).

2.5.3　Solid state form

In order to investigate the changes in the solid state of the drug and presence of amorphous state methods such as X-ray and DSC analysis can be used. Methods are described in Table 5 (Peltonen, L., et.al., 2015).

Table 5. Methods of nanocrystals solid state analysis

Method	Description	Information about
DSC (Differetial Scanning Calorimetry)	change in heat flow resulting from sample changes during heating /cooling	polymorphic form: melting point, crystallization temperature, Amorphous form: glass transition temperature Crystallinity: enthalphy, heat capacity change at T_g measures melting point, fusion entalphy of the mixture as well as raw drug and drug nanocrystals, the decrease in drug nanocrystal melting point and fusion enthalpy means a reduced crystallinity and thus adequate dispersion of the drug in the stabilizers.
XRPD (X-ray Powder Diffraction)	Diffraction of x-rays from lattice planes	polymorphic form (unique diffraction peaks), amorphous form (no peaks)

Understanding and investigating the polymorphic changes play an important role during nanosizing process, especially the conversion between amorphous and crystalline state.

2.5.4 Toxicity

Up to date, nanocrystals are considered as safe and well tolerated but it is still subject to investigation. There are always some potential issues and undesirable therapeutic effect which can occur when delivered through any route (oral, through respiratory system, blood circulation as well as administration through skin barrier) resulting from their nanosized character. E.g. they have a potential to induce oxidative stress, impair cell viability, phagocytosis or cell proliferation as well as remain in the body for a significantly longer period. Usually, particles with size ranging from 100 nm to 1000 nm are phagocyted by monoculear phagocyte system, however, particles smaller than 100 nm

may be taken up by all cells in the body. We can divide all nanocrystals into four classes according to the **NCS** (nanotoxicological classification system) as shown in the following Table 6 (Leslie Pelecky, D. L., 2007; Müller et al., 2011).

Table 6. Nanotoxicological Classification System

NCS class	Particle size and biodegradability of particles
Class l	> 100 nm, biodegradable
Class ll	> 100 nm, non-biodegradable
Class lll	< 100 nm, biodegradable
Class lV	< 100 nm, non-biodegradable

It should be taken into account, that each drug particle dissolving in the GIT will move from „ μm" to „ nm" size in the dissolution process. Considering this outline, drug nanocrystals can be considered as safe, as far they are aimed to be dissolved and not to accumulate in human body.

2.6 Supersaturated state

As mentioned earlier, the solubility of the most stable crystalline state of the chemical entity in a certain medium (at defined temperature and pressure) can be defined as thermodynamic solubility. The degree of solubility of a compound in a specific solvent is measured as the saturation concentration (C_s). However, under certain conditions, solubility can be increased as in the amorphous state, metastable polymorphic states, cyclodextrin complexes, as well as in nanosized particles. There are several terms for this solubility including kinetic, metastable or apparent solubility. In other words, apparent solubility is the solubility of less thermodynamically stable systems, described as a maximum achievable concentration in drug supersaturated state (Peltonen, L., et al., 2015).

According to Kelvin and Ostwald-Freundlich equation mentioned before, nanosized particles are capable to increase apparent solubility until certain fold. (Müller R.H., et Junghanns, 2008). Consequently, when the apparent solubility of nanocrystals is higher

than thermodynamic solubility, the supersaturated state in solution appears, defined also as ‚spring effect'.

Application of high energy forms to provide supersaturated solutions of poorly water soluble drugs has found to be a successful approach how to improve drug delivery. In supersaturated state (as a state in which thermodynamic activity is increased), the driving force for transport across the biological membranes is enhanced. (Ilevbare, G. A., et al., 2013). This is provided by the higher concentration gradient of the drug at the place of its action and subsequently acceleration of passive diffusion.

On the other hand, due to thermodynamic instability resulting in spontaneous crystallization, maintenance of supersaturated state in GIT for an appropriately long time, may be difficult.

Precipitation is the phase separation of solid material from a solution (but also from melt or gas phase). If the physical form formed is unknown, the term precipitate (including both crystalline and amorphous material) can be used.

The solution has to be supersaturated with regard to the solute in order to undergo crystallization and this process leads to thermodynamically favored state. However, the supersaturation state is not sufficient to initiate nucleation and nuclei and seeds are required. Thus, under certain circumstances, this crucial step can be delayed even though the crystallization process is thermodynamically beneficial for the system. The period between the supersaturation state and initiation of nucleation is called induction period and the length of this period is decreased by the presence of impurities or seeds, as well as increased supersaturation. The process of crystallization is complex and to date, not fully understood but in general accelerated by following factors:

- Increasing degree of supersaturation
- Increasing solubility (at constant supersaturation)
- Presence of impurities
- Lower temperature
- Low solution viscosity
- Decrease in the interfacial tension

Equation 4 shows the thermodynamic driving force in dilute solution, is governed by supersaturation ratio.

$$S = \frac{C}{C_{eq}}$$

Equation 4, where C stands for the concentration of the supersaturated solution and C_{eq} is the equilibrium solubility of the crystalline compound at a defined temperature.

Nucleation-induction time is another characteristic of crystal formation kinetics: **the true nucleation time** is the time that elapses from the moment of creation of supersaturation state until the formulation of nuclei.

However, the nuclei formation cannot be measured directly because they need to reach certain detectable size. On the other hand, it is possible to measure so-called **experimental nucleation time**, which is defined as the time that elapses between the moment of creation of supersaturation state and the first change in physical property (for example turbidity) which can be observed by adequate devices (light scattering). This characteristic strongly depends on the method used during detection. Classical nucleation theory (explained by equation 5) says that clusters enlarge on size until the critical size is reached, but there is no information about the structure of aggregates or about pathways leading to change from solution to crystalline solid.

$$J = A \exp\left(\frac{-\Delta G_{crit}}{kT}\right)$$

Equation 5. According to the classical nucleation theory J stands for nucleation rate, A is the pre-exponential kinetic factor, G_{crit} is free energy change, k is the Boltzmann constant, T is the absolute temperature.

According to the facts mentioned above, benefits assigned to transient supersaturation largely depends on the fact how fast the drug precipitates from the supersaturated state. Thus, it is important to maintain supersaturated state for long enough for desirable drug absorption to occur. In this field the term **„ spring and parachute effect"** is used,

referring to the creation of supersaturated state and subsequent inhibition of precipitation. This parachute effect can be caused by a combination of mechanisms. Stabilizers are able to themselves increase the thermodynamic solubility (co-solvency effect), resulting in decreasing supersaturation and thus driving force for crystallization. Even the small amounts of stabilizers (0.1 %–0.25 % w/w) are able to increase solubility in a significant way.

Stabilizers attached to the particle surface can also block the interactions of particles already dissolved, thus crystal growth. And third way of preventing crystal growth is due to viscosity of polymers solution and subsequent.

Several approaches for reducing the rate of drug precipitation from supersaturated solution has been taken to try, using precipitation polymer inhibitor included (described below).

2.7 Stabilization of nanocrystals

As mentioned above, thermodynamically and kinetically unstable system like nanocrystals needs to be stabilized by surfactants and/or polymers. However the relationship between efficacy of stabilizer and stability of nanosuspension has not been characterized enough, therefore stability problems present big limitations during the development of drug delivery system. A key problem is that there is no universal way to select an optimal stabilizer for drug nanosuspension because the stabilization efficiency depends on a variety of parameters: the interactions between drug and stabilizer, especially the functional group on stabilizer and drug surface; the amount of surface energy of drug; the viscosity of the solution; and most importantly, the affinity between stabilizer and drug. (Choi, et al., 2005; Van Eerdenbrugh et al., 2009).

In the absence of suitable stabilizer, the high-surface energy of nanocrystals results in agglomeration, aggregation and also may lead to phase separation. While agglomerates are held together by weak forces, aggregation forms stronger clusters which are usually irreversible.

Formation of larger particles may directly influence the dissolution velocity and thus *in vivo* performance of the drug, or even worse, it can cause vascular blockage after intravenous administration.

However, different stabilizers are required for different drugs and there are very little systematic understandings available for appropriate stabilizer selection. It is unrealistic to find a single universal stabilizer that properly works for all different wet comminution processes (Jonghwi L., et al., 2005). Usually, every compound has its own, most suitable stabilizer.

During the top-down method of preparation, freshly generated surface possesses high energy thus tends to agglomerate/aggregate in order to reduce Gibbs free energy in the system. On the other hand, during bottom up method, nucleation and crystal growth occur spontaneously in order to decrease free energy in the supersaturated solution. Unfortunately, trial and error approach is typically used, which is not efficient, as well as time-consuming.

Stabilizers are capable of spontaneous adsorption onto particle surface, provide wetting and cover this freshly generated surface. Hence this result in a decrease in free energy of the system and surface tension of particles.they are responsible for the formation of the dense hydrophilic layer around hydrophobic particles. In order to establish the effective stabilization, fast adsorption, full coverage, long desorption time is required.

Stabilization can be reached by two different mechanisms: either **steric hindrance** (polymeric, and non-ionic surfactants) or **electrostatic repulsion** (ionic stabilizers) as illustrated in figure 8. Also, the combination of both mechanisms is possible.
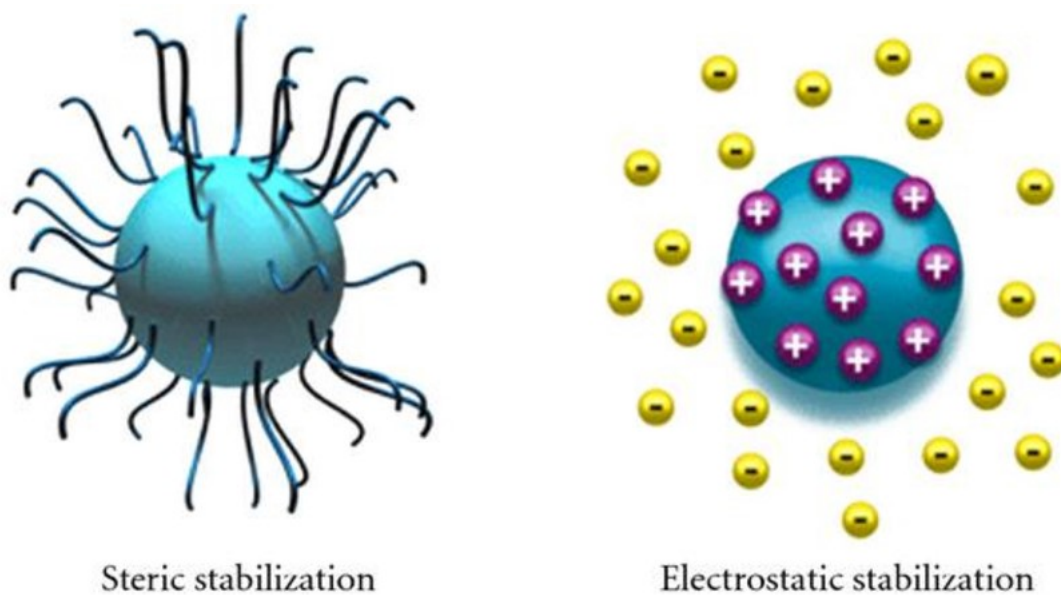

Steric stabilization   Electrostatic stabilization

Figure 8. Two types of stabilization, retrieved from Yu, L.X., et al., 1996.

Steric or electrostatic stabilization of used stabilizer is a result of its adsorption onto the surface of the particles by its hydrophobic moieties. The adsorbed chain molecules on the surface show unceasingly thermal motion, which results in dynamically rough surface providing against coalescence by repulsive entropic force. (Lee, J., et al., 2005). Electric stabilization is efficient in aqueous medium but shows some issues on dry formulations. Environmental factors determine the surface charge and, thus, electrostatic stabilization is diminished in presence of the other charged material (e.g. aqueous buffer solution used as a medium during milling).

Nonionic stabilizers efficacy is based on the steric action when long chains prevent the close contact between particles and hinder Van der Waals forces between them. Steric stabilization is shown to be sensitive to temperature changes.

Besides, as the molecular weight of the steric stabilizer increases, the adsorption rate decrease.

It is shown that polymeric-type steric stabilization is effective in providing drug stability during processing because compared to conventional low molecular weight surfactants, it does not usually destroy the crystal structure of the particles. Surfactants are also capable of creating the micelles containing a small number of dissolved molecules of the drug (Wang, Y., et al., 2013).

Ionic changes and pH also have an impact on stabilization. It is important to take into account all of the factors during drying and sterilizing for further formulation steps. (Uchegbu, I.F., et al., 2013*).* It is also important to choose adequate weight ratio of drug to stabilizer (usually 20:1 to 2:1) (Merisko-Liversidge, E., et.al., 2003).

The concentration of polymer plays an important role, because under certain concentration level micelle formation of polymer occurs, and thus, it can compete with surface adsorption. There is a small amount of drug which can be solubilized by creating micelles, but generally, micelle formation is an unwanted feature (Choi, et al., 2005). If there is too little stabilizer, aggregation/agglomeration can appear, on the other hand, too much stabilizer induces Ostwald ripening (Figure 8).
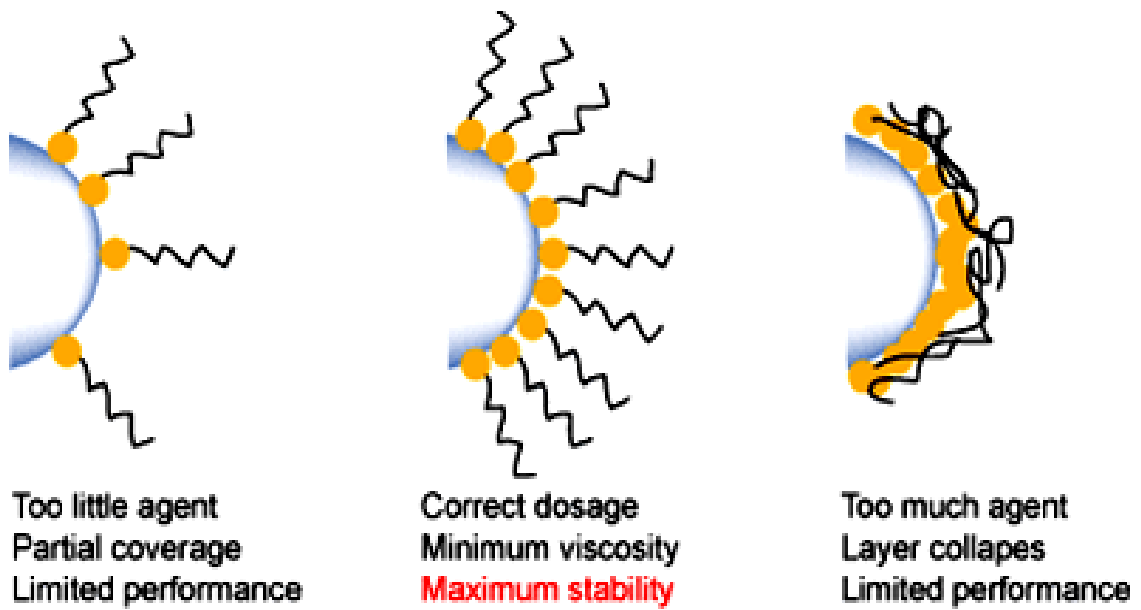
Figure 9. Correlation between amount of stabilizer and its performance, retrieved from http://www.uniqchem.com/?page_id=1083&lang=de [cited 2.5.2017]

The solubility of stabilizer in the medium also determinates efficiency of steric stabilization, therefore well-solvated stabilizers have a tendency to minimize particle interactions as shown in Figure 10.
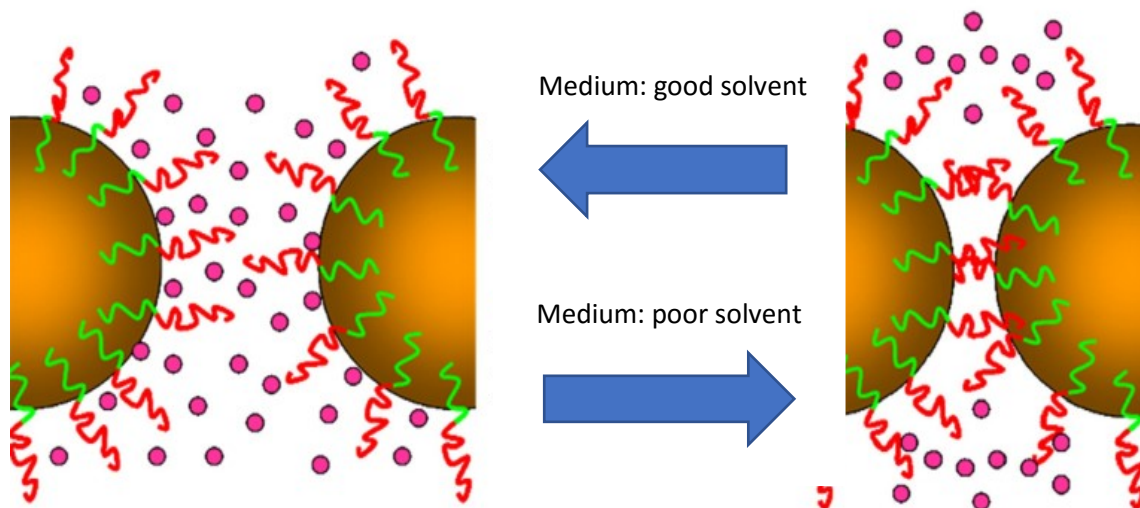

Figure 10. The choice of solvent as a determinant of stabilizers efficiency. If the medium is a good solvent for the stabilizing moiety, the adsobed layers on particles do not interpenetrate each other,

however in the case of medium, which is a poor solvent for stabilizer, particles may agglomerate. Retrieved from Wu, L., et al., 2011.

The molecular weight of stabilizer affect viscosity, and increased viscosity normally prolongs processing time. However, in the final formulation, it prevents the aggregation process. In general, increased viscosity results in some serious issues such as lower diffusion of stabilizer molecules, hindering of milling pearls performance as well as increasing in processing time (Dolenc, A., et al., 2009).

We should also take into account interactions between molecular functional groups of stabilizers and the drug. For example, appropriate stabilization may be also hindered due to the hydrogen bonding between the stabilizer and drug. Besides, also functional groups like amines, hydroxyls, carboxylic acids, and phenols are capable of interaction with stabilizer.

Other properties of stabilizers such as an impact on the cell membrane should be considered (for example, some stabilizers are capable of making the cell membrane leakier or stimulate P-gp activity) (Peltonen, L.,et al., 2015).
Currently, screening approaches to maintain suitable desirable stabilization are emprical, without sufficient understanding the interaction between nanoparticle surface and polymers. Stabilizers that have been explored so far include cellulose derivates, poloxamers, polysorbates, lecithins and povidones (Merisko-Liversidge, et al., 2003).
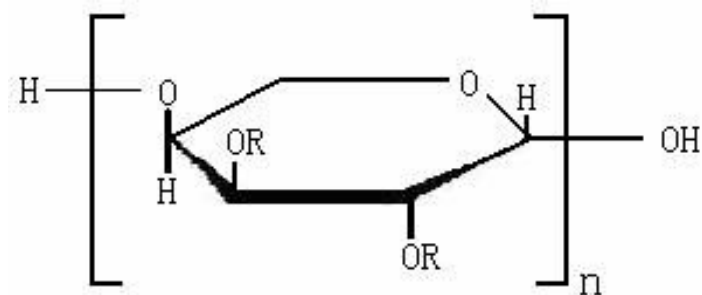
2.7.1 Hydroxypropyl methylcellulose

Figure 10. The universal chemical structure of hydroxypropyl methylcellulose. Where n is a number of glucose units in a cellulose molecule, R is CH₃ or CH₂CH(OH)CH₃ (Savage, A. B., 1965).

Hydroxypropylmethylculluloses are water soluble (10 mg/ml) polymers derived from cellulose, soluble in cold water, while insoluble in hot water as well as organic solvents (European Pharmacopoeia, 2014a).

In nanocrystal formulations, typically HPMCs with the lowest molecular weights are applied for stabilizing purposes. Attachment of HPMC on the particle surface is mainly based on hydrogen bonding. Molecules of HPMC consist of the high degree of substitution of the hydroxypropoxy and methoxy groups both capable of surface attachment. Based od earlier studies (Miller, D., et al., 2008), HPMC has been shown to prevent the precipitation/crystallization of ITZ.

2.7.2 Poloxamer 407

Poloxamer 407 is a synthetic block copolymer of ethylene oxide and propylene oxide, represented by the following formula (Figure 11). It is very soluble in water and in ethanol (96%) (European Pharmacopoeia, 2014b).

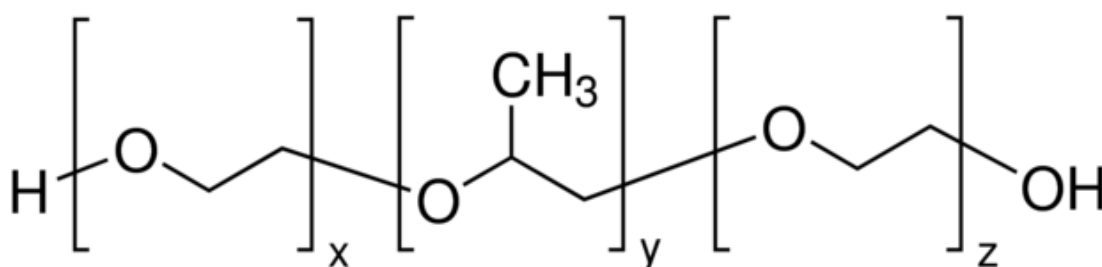*Figure 11. Chemical structure of Poloxamer 407, X= 101, y= 56, z=101*

Hydrophilic parts surround the molecule of drug and thus provide steric hindrance resulting in inhibition of aggregation and crystal growth. On the other hand, hydrophobic parts of polymer molecule are capable of hydrophobic interactions and thus adsorption onto crystal surface (Liu, P.; et.al., 2011, Tuomela, A.; et al., 2016).

2.8 Bioavailability after oral administration

Generally, oral administration is the most common and suitable route for drug delivery due to its significant benefits. Ability to formulate nanocrystalline drug may have a dramatic effect on bioavailability. Theoretically, if bioavailability is truly limited by dissolution rate, particle size reduction can significantly improve the performance of the drug. However, if orally administered, bioavailability also depends on drug permeability, first-pass metabolism as well as susceptibility to efflux mechanisms. (Khadka, P., et al., 2014) Bioavailability of a certain drug is defined as a rate at which the drug is absorbed in the desired site after administration. Absorption presents the transfer of a drug molecule from the administration site into the blood or lymphatic circulation. The amount of absorbed drug is determined by physicochemical properties of the drug, pharmaceutical dosage form, and physiological conditions of the absorption site. In the body, passive diffusion is considered to be the main mechanism of drug absorption. Before transporting through the gut wall, the drug needs to be dissolved. (Dizaj S.M., et al., 2015).

As mentioned above, poor water solubility and low dissolution velocity in human GIT can result in a large amount of undissolved drug and thus the absorption window and low BAV. Besides, high drug dose is required which can cause undesirable side effects to the tissue, poor patient compliance and high-priced therapy. Table 7 depicts some important advantages of nanocrystals when taking orally (Liversidge, G.G., et al., 1995; Jia, L., et al., 2002; Merisko-Liversidge, E., 2003; Jacobs, C., et al., 2001).

Advantage:
Higher peak concentration (C_{max}) of the drug in plasma
Faster onset of action
Faster dissolution in narrow absorption window
Decreased fed-fasted variation
Improved dose proportionality
Ability to be formulated into various pharmaceutical dosage forms (tablets, grain filled capsules, pellets, and oral suspensions.
decreased gastric irritancy

Table 7. The important advantages of nanocrystals orally administered.

3. Experimental part

The experimental part of this experiment is divided into two parts:

During the first part, when nanosuspension was prepared, poloxamer 407 (Lutrol F127) was added before milling, while hydroxypropyl methylcellulose (HPMC) right after the milling process. During the second part, both stabilizers were added directly before the milling process.

3.1 Chemicals

In this study, itraconazole (Orion Pharma, Espoo, Finland) was used as a model drug. Hydroxypropyl methycellulose (Methocel Premium LV EP, type 2910) from The Dow Chemical Company (Midland, Michigan, USA), and poloxamer 407 (Lutrol F 127) from BASF Co. (Ludwigshafen, Germany) were used as stabilizers. Methanol (Methanol anhydrous 99.8 %, Sigma-Aldrich, Steinheim, Germany) and hydrochloric acid buffer (pH 1.2, hydrochloric acid 37 %, Riedel-de Haen, Seelze, Germany and potassium chloride, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) were used as a media.the water used was ultra purified Milli-Q® water (Millipore SAS, Molsheim, France).

3.2 Methods
3.2.1 Media milling

Rapid Top Down, Wet-media milling technique was chosen as a preparation method of nanosuspension. The HPMC (0.4 g) was dissolved in 5 ml of water and the F127 stabilizer (1.2 g) was dissolved in 10 ml of water, thereafter both were shaking on an orbital shaker overnight.

Next, **during the first part** of the study, a suitable amount of solution of F127 was added to 2g of bulk ITZ and mixed firmly in a beaker. The ITZ suspension prepared in this way was poured into milling vessel and the rest of the solution was used to collect the residual suspension from the beaker.

45 ml volume vessel was filled with 70 g of milling pearls - dimeter 1 mm, zirconium oxide).

A planetary ball mill (Pulverisette 7 Premium, Fritsch Co., Idar-Oberstein, Germany) was used for the milling. Grinding was performed at 1100 rpm during 10 cycles lasting 3 min. After each milling cycle, there was 15 min pause for the system to cool down in order to avoid warming up of the sample. The rotational direction was not reversed during the milling. After the milling process, the nanosuspension was collected by pipetting into the vial in order to separate milling pearls from the nanosuspension. The solution of HPMC was added into this nanosuspension in the end.

During the second part of the study, both stabilizers (F127 and HPMC) solution were added directly to the milling vessel before the milling process.

The nanosuspension was used for further studies as such.

3.2.2 Dynamic light scattering

The mean particle size and polydispersity index (PDI) of the nanosuspension were analyzed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 3000HS (Malvern Instruments, UK).

The Dispersion Technology Software DTS (Malvern Instruments Ltd, UK) was used to calculate the mean hydrodynamic particle diameter expressed as Z-average (nm) and the polydispersity index PDI. The samples were measured in triplicate.

Measurements were performed from fresh nanosuspensions. Before measurement, the saturated solution of ITZ was prepared in order to prevent nanoparticles from dissolving.

The day before the DLS measurement, the **saturated solution** was prepared. Saturated solution contained 0.1 g of F127, 0.1g of HPMC and an excess amount of ITZ in order to achieve complete saturation. All these components were dissolved in Milli-Q water and placed into orbital shaker overnight. Prior using, the saturated solution needed to be filtered by using 0,45 µm Acrodisc® Syringe filter with GHP Membrane (Pall Corporation, New York, USA).

For DLS particle size measurement, the nanosuspension sample of saturated solution was used for preparation of diluted medium. Such diluted medium consisted of 0.1 g nanosuspension and 1.9 ml of saturated solution mentioned above (i.e. 20x dilution). This 20 times dilution served for further dilutions when necessary. In order to reach required dilution level, count rate was checked by Zetasizer software before the measurement. Accordingly, the sample used for measurement was prepared from this 20-time dilution (100 µl of dilution was taken) and diluted with further amount of saturated solution (5 ml).

The measurement (n = 3) was composed of three running cycles consecutively and the average of these three measurements was presented as a final information about nanosuspension characterization.

3.2.3 Preparation of standard solutions

Accurately weighed quantity of 10 mg ITZ was transferred into 50 ml volumetric flask, dissolved and diluted up to the mark with methanol to give a stock solution having concentration of 200 µg/ml.

0, 2 µg/ml, 1µg/ml working standard solutions were prepared by diluting 50 µl, 250 µl of stock solution to 50 ml with methanol respectively. Further, 2 µg/ml, 5µg/ml and 15 µg/ml working standard solutions were prepared by diluting 200µl, 500 µl and 1500 µl of stock solution to 20 ml with methanol respectively.

The calibration curve of absorbance vs. concentration was plotted, correlation coefficient and regression line equation for ITZ were determined (Figure 10). The calibration curve was measured from a series of standard solutions that had been prepared from a stock solution of ITZ (concentration 200 µg/ ml). Concentrations of standard solutions were: 0, 2 µg/ml, 1µg/ml, 2µg/ml, 5µg/ml, and 15 µg/ml. The wavelength 262 nm was used for the measuring the calibration curve. According to the calibration curve, the following equation was obtained: $y = 0{,}0441\ x - 0{,}0014$,
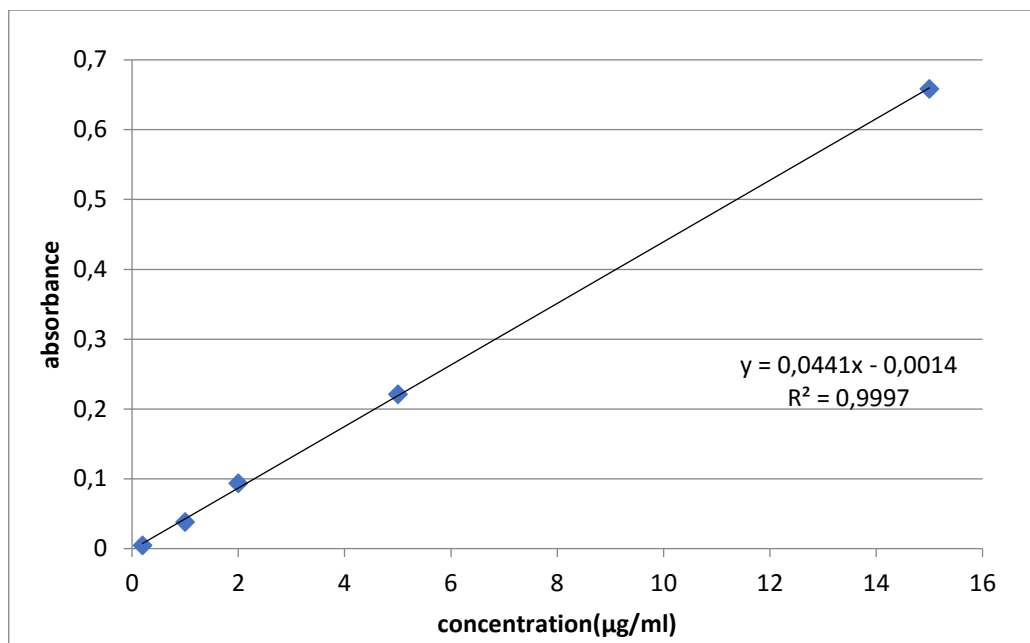
$$r^2 = 0{,}9997.$$

Figure 10. Calibration curve of Itraconazole by simple UV spectroscopy

The concentration of ITZ was determined by using UV spectrophotometer (UV-1600 Pc Spectrophotometer VWR International BVBA, Leuven, Belgium). Results were evaluated using M. Wave Professional 1.0 software. Quartz cuvette with 1 cm light path was used. In order to eliminate errors, only one cuvette was used throughout the whole experiment.

3.2.4 Preparation of buffer solution

Buffer solution used as a medium was prepared according to US Pharmacopoeia (The United States Pharmacopoeia USP 23 – National Formulary NF 18, 1994).

3.2.5 Method development
The first part

In order to achieve the sample concentration about 12.5 µg/ml, approximately 50 µl of freshly prepared nanosuspension of ITZ was pipetted and diluted with 200 ml of HCl buffer of pH 1.2. This way of preparation was used throughout this work. The solutions

were always prepared in duplicate (**solution A** for checking up of concentration, **solution B** for the method measurement itself).

Right after preparing solution A, the sample of 1 ml was inserted into the sample holder of UV spectrophotometer and absorbance was measured at the wavelength 262 nm to check the required concentration. As the concentration was confirmed, the wavelength was changed to 551 nm. Absorbance at 551 nm of freshly prepared solution B was then measured for 120 minutes. This long time investigation served as a proof that no changes in absorbance values occurred.

Absorbance of the blank reference (i.e. buffer solution) before each analysis was measured as well. Sample concentrations are presented in   Table 8:

Sample number	Absorbance at the wavelength 262 nm	Concentration
1	0.6504	14.78 µg/ml
2	0.502	11.43 µg/ml
3	0.5616	12.77 µg/ml

Table 8. Sample concentrations achieved in the first part of the study.

As far as the result of the sample number 1 analysis verified no changes in the sample, measurement after stressing with different pH could be examined.

The environment with pH 7 was achieved experimentally and checked with universal pH indicator before every measurement. The proper amount of NaOH needed to change pH to desired values was found experimentally before the experiment beginning. When pH 7 value was reached, the sample (1 ml) was immediately measured. In order to reduce errors arising from delayed start of the measurement, the proper amount of NaOH solution was added directly to the cuvette filled with the sample ready for the measurement. As desirable pH was achieved, a cuvette cap and fast shake up were done to assure the same concentration through the sample.

The absorbance values at 551 nm were measured every minute during 120 minutes. Three subsequent measurements were carried out.

Two additional measurements of stability of nanosuspension without stressing were done (sample number 2 and sample number 3) in order to prove that no changes occurred within three samples.

The second part

The dilutions of nanosuspension were prepared in the same way (50 µg/ml of nanosuspension and 200 ml of buffer), in order to achieve concentration ranging from 10 µg/ml to 15µg/ml, and three different concentration levels were reached (Table 9).

Sample number	Absorbance at the wavelength 262 nm	Concentration
1	0.656	14.91 µg/ml
2	0.475	10.80 µg/ml
3	0.628	14.27 µg/ml

Table 9. Sample concentrations achieved in the second part of the study.

In this part, the analysis of stressed sample was done right after every single sample was prepared.

4. Results and discussion

4.1 Choice of proper solvent

Due to a poor aqueous solubility of ITZ, methanol was chosen as a solvent medium for standard solutions in order to be able to prepare concentrated solutions. When choosing a suitable solvent, one should be aware of its absorbance cutoff wavelength, below which the solvent itself absorbs. The absorbance cutoff wavelength of methanol is 205 nm. As it is not close to the wavelength where ITZ absorbs (262 nm), we may use methanol as a solvent. As a cuvette for UV spectroscopy, the one made of quartz is required (Worsfold, P.J., 1993).

4.2 Choice of sample concentration

Based on previous experiments, the most suitable concentration of ITZ was found to be between the values of 10 µg/ml and 15 µg/ml, in order to achieve the sample solution of approximate concentration 12,5 µg /ml and thus obtain the best possible results. (Kubačková J., 2016).

4.3 Determination of suitable wavelengths

The wavelength where ITZ has its absorbance maximum is represented by the value of 262 nm. In order to carry out this new method, the wavelength value where dissolved ITZ does not show any signals needed to be investigated. This was done experimentally using UV spectrophotometer. Approximate expected value was about 550 nm based on previous experiments with indomethacin nanocrystal preparation UV imagining investigated by Sarnes A., et al., 2013. The absorbance of ITZ nanosuspension which was the nearest to zero was found to be 551 nm. Subsequently, this value was used in this method.

4.4 Choice of stabilizing agents

In this study, two stabilizing agents were used: Poloxamer 407 (F127) and hydroxypropyl methylcellulose (HPMC). Addition of F127 provides effective steric stabilization of nanosuspension and hinders particle aggregation. The second stabilizer, HPMC helps to maintain supersaturation state when drug nanocrystals are dissolved.

Poloxamer 407 (F127) represents a non-ionic linear triblock copolymer group of stabilizers, consisting of a hydrophobic central PPO segment and two hydrophilic side PEO segments. Hydrophilic parts surround the molecule of drug and thus provide steric hindrance resulting in inhibition of aggregation and crystal growth. On the other hand, hydrophobic parts of polymer molecule are capable of hydrophobic interactions and thus adsorption onto crystal surface. Several studies have shown that poloxamers such as F127 are suitable as ITZ nanocrystal suspensions (Liu, P.; et.al., 2011, Tuomela, A.; et al., 2016).

Hydroxypropyl methylcellulose is semisynthetic non-ionic polymer derivated from cellulose and widely utilized not only as a stabilizer but also generally in pharmaceutical applications because of its natural origin (non-toxic, non-irritating properties). In nanocrystal formulations, typically HPMCs with the lowest molecular weights are applied for stabilizing purposes. Attachment of HPMC on the particle surface is mainly based on hydrogen bonding. Molecules of HPMC consist of the high degree of substitution of the hydroxypropoxy and methoxy groups both capable of surface attachment. Based od earlier studies, HPMC has been shown to prevent the precipitation/crystallization of ITZ (Miller, D., et al., 2008).

Only one stabilizers formulation, was applied in this study, based on previous work of Kubačková, J., 2016. In this work screening of various combinations of stabilizer was carried out. It was found that combination of F 127 and HPMC with their concentration with respect to drug amount 60 % and 20 % respectively, was shown to be one of the best composition and, thus, suitable for this study.

The second stabiliser, the stabiliser with positive impact on supersaturated state maintenance, can be added predissolved in water before the milling or predissolved in solubility testing medium after milling.

The stage at which the stabilisers are added determinates their interactions on surface of poorly soluble drug. In previous studies, it has been shown that various addition stage of the predissolved second stabilizer may influence the precipitation behavior of ITZ (when adding second stabilizer before milling, parachute effect was missing). However, in **this study** no differences were noticed in the precipitation behavior, precipitation took quite the same time, undependent if HPMC was added before or after the milling. Slight differences between PDI exist when second stabilizer added before or after milling, but it is difficult to say how much it affects the precipitation results.

4.5 Evaluation of particle size

ITZ nanosuspensions were successfully prepared, however with the same stabilizers:drug ratio, results quite differs from each other. Particle size is considered one of the most important parameters characterizing the stability of nanosuspension. Therefore, in order to evaluate the condition of freshly prepared nanosuspension, particle size needed to be investigated.

Nanosuspension with PDI lower than 0.2 indicates monodisperse particles within the sample. This is the case of the second part. Particle size distribution is considered broad in the first part, as the values of PDI lies between 0.5 and 0.7. Polydisperse systems have PDI values above the 0.7. (Müller and Jacobs., 2002).

The experiment was carried out with the sample of ITZ nanosuspension which was diluted before, to reach count rate required for particle size measurement (ideal count rate ranges from 200 pcs to 600 pcs). Compared to the previous experiment focused on stabilizers of nanocrystal suspensions, comparable results were obtained, to be specific Z-average: 325 nm and PDI: 0, 166. (The same milling conditions used) (Kubačková, J., 2016).

Figure 12 depicts signals obtained by measurement of nanosuspension prepared by adding F127 stabilizer solution before milling and HPMC stabilizer solution after milling.

Figure 13 was obtained in the same way but this time it depicts parameters of nanosuspension prepared by adding both stabilizer solutions before milling - directly to the milling vessel.

On the first graph (Figure 12), we can see the main peak but region of this peak does not reach 100 %. However, the second peak is considerably small as compared to the first peak. On the other hand, we can see only one main peak on the second graph (Figure 13). In both cases, the sample was measured three times, resulting in following average graphs. Nanoscale was achieved in both samples.
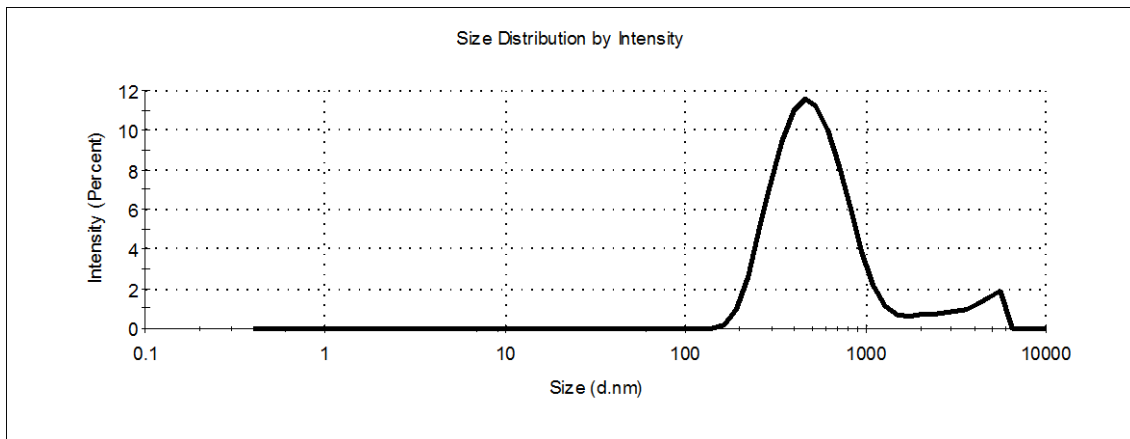

Figure 12. Photon correlation spectroscopy of ITZ nanosuspension of the first part.

Count rate (kcps): 226.4

Z-average (d.nm): 487.1

PDI: 0.317

Peak 1 size: 540.2, intesity: 90.9 %   Peak 2 size: 3809, intensity 9.1%
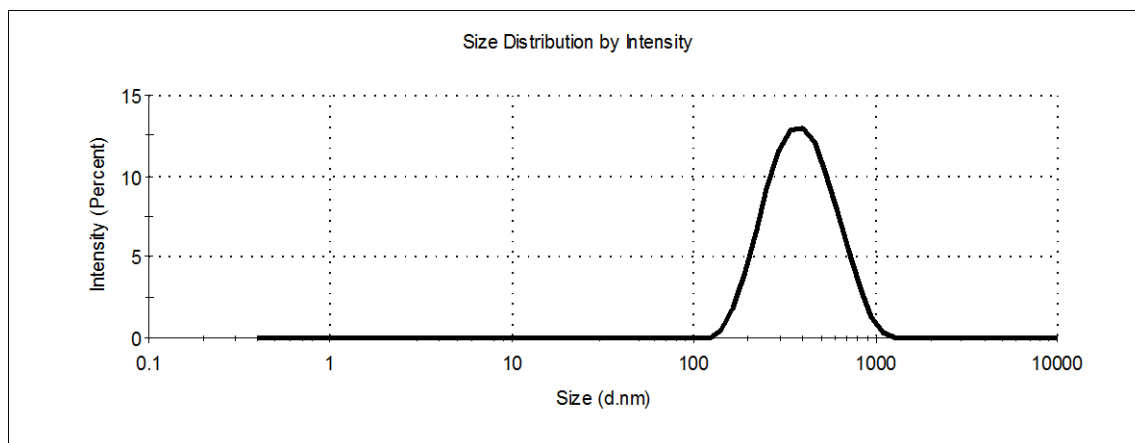
Figure 13. Photon correlation spectroscopy of ITZ nanosuspension of the second part.

Count rate (kcps): 399.6

Z-average (d.nm): 355.1

PDI: 0.167

Peak 1 size: 417.9, intensity: 100 %

Both samples were evaluated by analyzing software as samples of good quality. Despite these different physical properties such as particle size and polydispersity index, prepared samples were both acceptable for using in the following analysis.

4.6 Evaluation of method

The aim of this experiment was to investigate the correlation between changes in UV absorbance of ITZ sample and crystallization process within the sample, by very simple and fast method.

As mentioned in Theoretical part, drug nanocrystals after oral administration occur in supersaturated state resulting in improved dissolution rate and thus bioavailability. However, the supersaturated state should be maintained for long enough for desirable drug absorption to occur. In the case of oral administration, an amount of dissolved drug is crucial to be provided in the main area of absorption – the small intestines. Various dissolution studies have shown benefits of drug nanocrystals orally taken, but this often fails when investigated *in vivo.* Thus, the control of the crystallization processes is crucial in pharmaceutical drug formulations.

Using UV spectrophotometer was suggested to be a valuable way to demonstrate and compare the dissolution behavior of nanocrystals. At the very beginning of this experiment, the absorbance value where dissolved ITZ does not absorb light was experimentally found to be 551 nm. Accordingly, if absorption is noticed at this wavelength, it means that the absorbance is due to precipitated/crystallized drug particles.

The wavelength 262 nm was used to verify the concentration of the sample as far as it is a well-known value where ITZ has its own absorbtion maximum. As a simple tool to calculate concentrations of samples, the calibration curve of standard solutions of ITZ was used. For this purpose, two dilutions were prepared before every measurement: somultion A to verify that ITZ concentration ranges from 10 µg/ml to 15 µg/ml, solution B, prepared in the same way for the measurement itself. As a dilution medium, HCl buffer pH 1.2 was chosen in order to achieve conditions similar to those in the stomach. The samples were measured three times for 120 minutes at 551 nm and during this time no increase in signal intensity was investigated. These indicate that the crystallization process did not occur (Figure 14 and Figure 15).
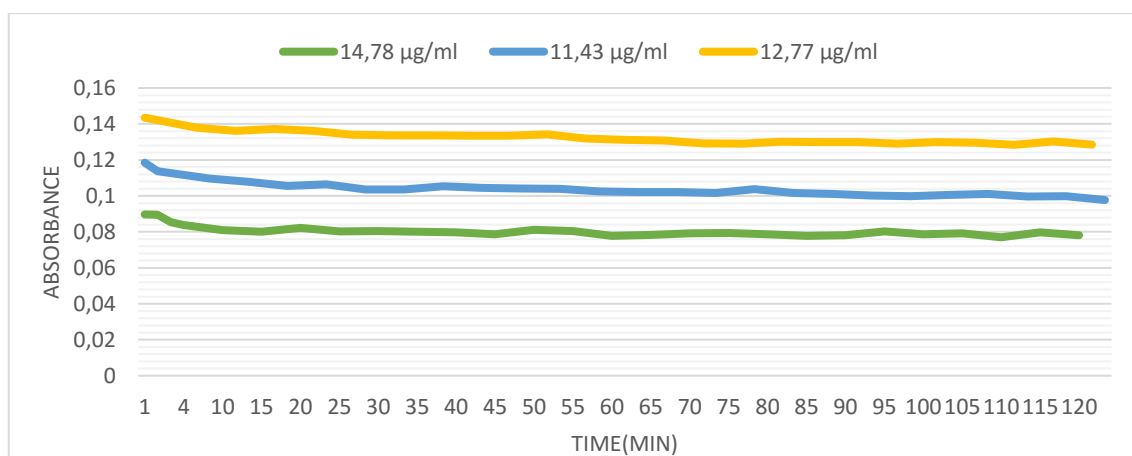

Figure 14. Measurement of ITZ nanocrystals samples of the first part, dissolution medium simulating the acidic stomach environment
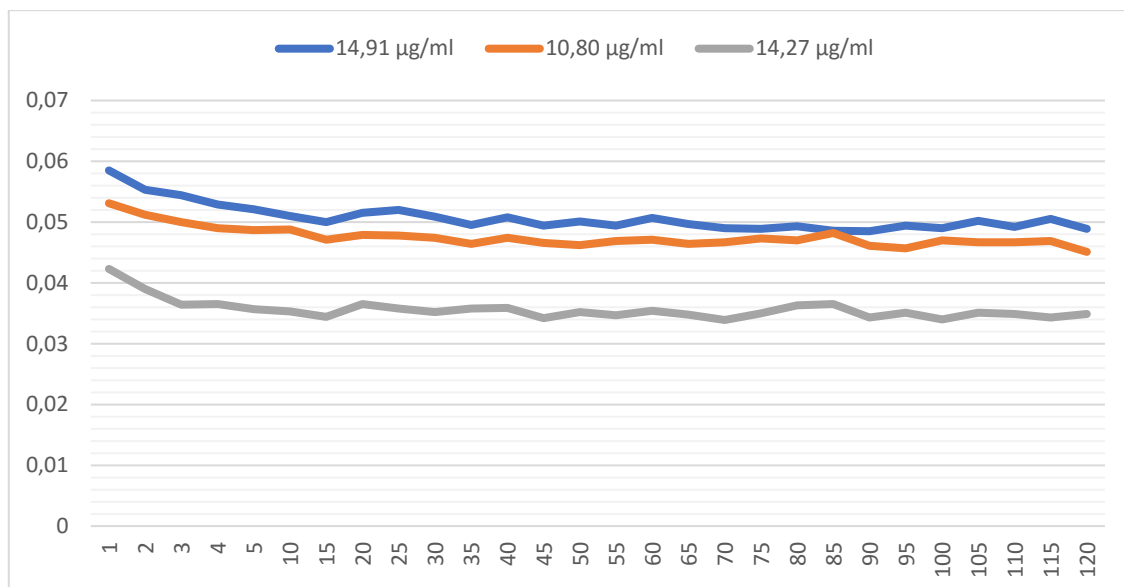
Figure 15. Measurement of ITZ nanocrystals samples of the second part, dissolution medium simulating the acidic stomach environment.

Both graphs depict no increase in absorbance during 120-minute measurement. In order to consider this simple method as reliable enough, the stressful environment had to be induced to see changes in absorbance, therefore a valuable asset of this method could be confirmed.

The solubility of ITZ as an extremely weak base, is significantly higher in the ionized form (when orally administered, presented in the stomach fluids) than in the unionized form. (after emptying from the stomach to the small intestine where, pH of environment is markedly higher). This represents an issue as far as ITZ nanocrystals when reaching the small intestines, tend to undergo a crystallization process, thus the absorption is decreased. In order to simulate described situation, HCl buffer with pH 1.2 was employed to represent acidic stomach fluids. As an illustration of the situation, when drug nanosuspension reaches the small intestine, NaOH was added, until pH 7 was reached. This NaOH addition had to be fast and carried out directly into the cuvette with the prepared sample, to eliminate errors resulting from the time delay of the measurement. Figures 16 and 17 clearly depict how fast the absorbance increases after pH stressing of samples.
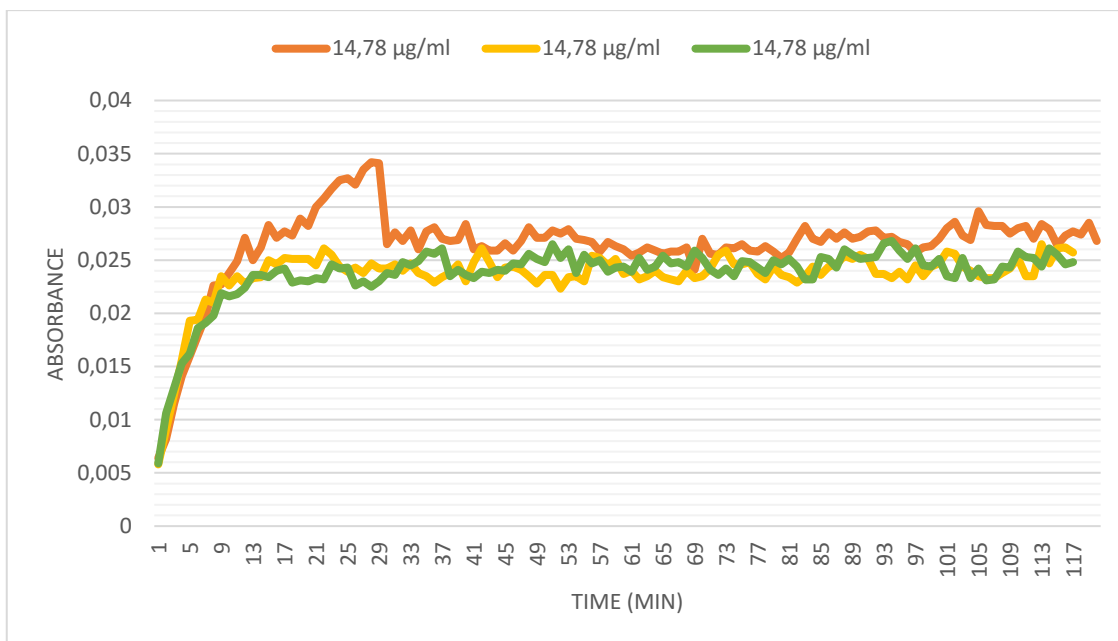
Figure 16. Measurement of ITZ nanocrystals samples of the first part, simulation of the small intestine environment.
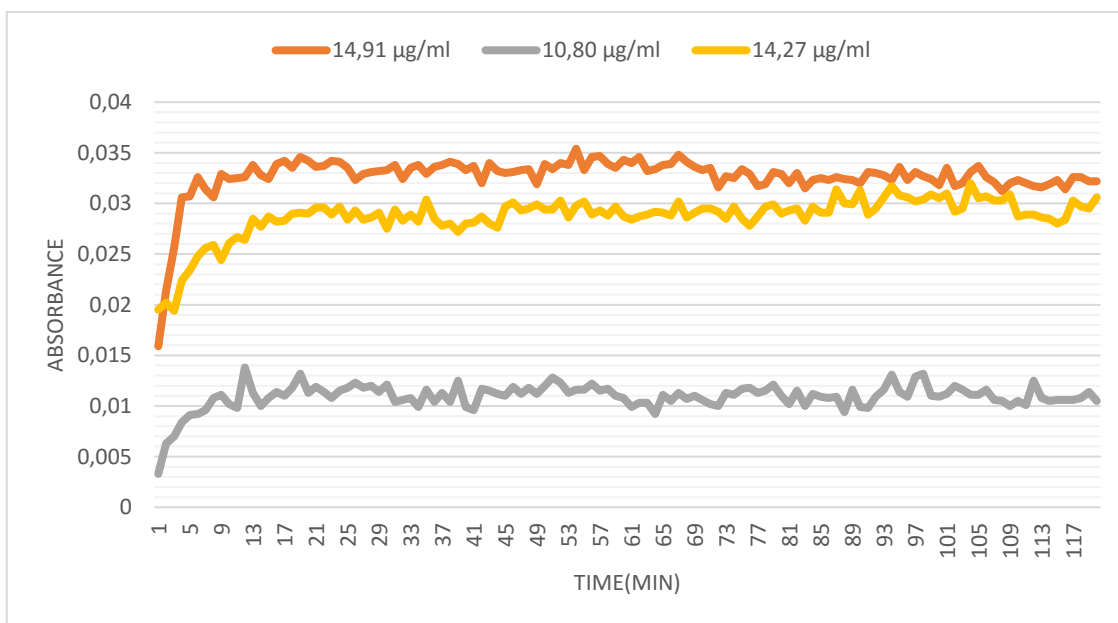

Figure 17. Measurement of ITZ nanocrystals samples of the second part, simulation of the small intestine environment.

In both cases, rapid increase in absorbance can be seen, until the critical time (5 – 10 minutes), after which the absorbance values are relatively stabilized.

In summary, the method tested here showed succesfully the precipitation behavior and with this technique it is possible to detect the precipitation with very small sample amount, sample preparation process is not time-consuming, and measurement is very fast as well.

5. Conclusion

In order to study the stability of metastable forms like drug nanocrystals, itraconazole (ITZ) nanosuspension was prepared by wet milling method of preparation.

With the new method tested here, it was possible to detect the precipitation behavior of drug nanocrystals by simulating the conditions similar to those in GI tract. In this fast technique, only a small sample amount was needed, and it was easy to change the measurement environment, e.g. to change the pH value.

6. References

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