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IZDELAVA NANOKRISTALOV GLIBENKLAMIDA Z MOKRIM MLETJEM V KROGLIČNEM MLINU

PREPARATION OF GLIBENCLAMIDE NANOCRYSTALS BY PEARL MILLING

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Magistrsko nalogo sem opravljala na Erazmus izmenjavi na University of Helsinki, Faculty of Pharmacy pod mentorstvom doc. dr. Petre Kocbek in somentorstvom doc. dr. Leene Peltonen. Analize nelinearnega optičnega slikanja so izvedli na Biomedicum Imaging Unit, Faculty of Medicine, University of Helsinki.

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TABLE OF CONTENTS

POVZETEK

Vse več novo odkritih zdravilnih učinkovin je slabo vodotopnih s posledično nizko biološko uporabnostjo. Topnost in hitrost raztapljanja lahko povečamo z zmanjševanjem velikosti delcev, kar predstavlja učinkovit in široko uporaben pristop, ki ni specifičen za določeno učinkovino. Eden izmed preprostih dostavnih sistemov, ki ga pripravimo z zmanjševanjem velikosti delcev v submikrometrsko območje, so nanokristali. To so nanometrski delci kristalne učinkovine, ki jih obdaja plast stabilizatorja. Različne metode za pripravo nanokristalov so predstavljene v uvodu, v raziskovalnem delu pa smo izmed metod, ki so na voljo, uporabili mletje v krogličnem mlinu. Kot modelno težko topno učinkovino smo izbrali glibenklamid.

Sprva smo preizkusili dva stabilizatorja (Poloxamer 188 in hidroksipropilmetilcelulozo (HPMC)), saj je izbira ustreznega stabilizatorja bistvenega pomena pri doseganju delcev nanometrske velikosti z zadostno dolgotrajno stabilnostjo. Izdelali smo serije z različno vsebnostjo Poloxamera 188 kot stabilizatorja in jih nato ovrednotili. Vsi nanokristali so bili homogeni (polidisperzni indeks okrog 0,2) s povprečno velikostjo, manjšo od 300 nm in z zeta-potencialom okrog –25 mV. Glibenklamid je bil po mletju še vedno v kristalni obliki, kar smo dokazali z diferencialno dinamično kalorimetrijo in rentgensko praškovno difrakcijo. Med procesom mletja ni prišlo do kemične razgradnje, kar smo potrdili s tekočinsko kromatografijo visoke ločljivosti. Nanokristale smo izdelali v obliki nanosuspenzij in jih nato posušili z liofilizacijo. Med sušenjem je prišlo do tvorbe agregatov, kar smo ugotovili z vrstično elektronsko mikroskopijo. Agregati se ob dodatku medija niso redispergirali nazaj v delce nanometrske velikosti, zato smo kot krioprotektant dodali laktozo.

Da bi dokazali povečanje hitrosti raztapljanja glibenklamida, ko je le-ta v obliki nanokristalov, smo uporabili farmakopejsko metodo raztapljanja z vesli. S pretočno celico smo potrdili povečanje topnosti, na kar vpliva tako nanometrska velikost delcev, kot tudi prisotnost Poloxamera 188. Povprečna velikost delcev in polidisperzni indeks sta ostala skoraj nespremenjena v obdobju 2 mesecev, kar nakazuje dobro fizikalno stabilnost formulacije.

Z izdelavo nanokristalov glibenklamida z mokrim mletjem v krogličnem mlinu smo dosegli izboljšanje topnosti in hitrosti raztapljanja te učinkovine, kar je bil glavni cilj naše raziskave.

KLJUČNE BESEDE:

Povečevanje topnosti • glibenklamid • nanokristali • mokro mletje v krogličnem mlinu

ABSTRACT

More and more newly discovered drug candidates are poorly water soluble resulting in low oral bioavailability. Particle size reduction is an effective approach of improving drug water solubility and dissolution rate, which is not drug specific. One simple drug delivery system prepared by reduction of particle size are nanocrystals, consisting of nanosized drug particles surrounded by a stabilizing layer. Different techniques for preparation of nanocrystals are briefly presented in the introduction; however, pearl milling has been applied in our research. Glibenclamide, poorly soluble hypoglycemic drug, was used as a model compound.

Initially two different stabilizers (Poloxamer 188 and hydroxypropyl methyl cellulose (HPMC)) were tested, since a proper stabilizing agent is of crucial importance for achieving nanosized particles with sufficient long-term stability. Batches containing different amounts of Poloxamer 188 as stabilizer were produced and further characterized. All nanocrystal formulations exerted homogeneous (polydispersity index around 0.2) size distribution, average size smaller than 300 nm and zeta potential around –25 mV. After milling, glibenclamide was still in crystalline state according to differential scanning calorimetry and X-ray powder diffraction measurements. There was no chemical degradation during milling process, as confirmed by high performance liquid chromatography. Nanocrystals were produced as nanosuspensions and later freeze-dried. Scanning electron microscopy showed presence of aggregates which had been formed during drying process. When medium was added, they did not redisperse to obtain nanosized particles. To overcome this problem lactose was added as a cryoprotectant.

Pharmacopoeian paddle method was performed to prove the enhancement in glibenclamide dissolution rate when it was transformed in nanocrystalline formulation. Solubility of glibenclamide also improved, as confirmed by flow-through cell measurement, due to the formation of nanosized particles and due to the presence of Poloxamer 188. Average particle size and polydispersity index of nanocrystalline formulations remained almost unaltered during 2 months period, indicating good formulation physical stability.

The primary aim of our study, the improvement in solubility and dissolution rate of glibenclamide, has been successfully achieved by formation of drug nanocrystals with pearl milling technique.

KEY WORDS: Solubility enhancement • glibenclamide • nanocrystals • pearl milling

RAZŠIRJENI POVZETEK

Vse več novo odkritih učinkovin je slabo vodotopnih, kar zmanjšuje njihovo biološko uporabnost. Topnost in hitrost raztapljanja lahko povečamo z zmanjševanjem velikosti delcev v submikrometrsko območje, kar predstavlja učinkovit in nespecifičen pristop. Eden izmed preprostih sistemov za dostavo učinkovin, ki ga pripravimo z zmanjševanjem velikosti delcev učinkovine, so nanokristali. To so nanometrski delci kristalne učinkovine, ki jih obdaja plast stabilizatorja. Izbor ustreznega stabilizatorja je bistvenega pomena, če želimo izdelati dolgoročno stabilni nanodostavni sistem. Nanokristale lahko pripravimo z različnimi metodami, ki jih razdelimo na "bottom-up" in "top-down" metode. Pri "bottom-up" metodah pripravimo nanometrske delce iz raztopljene učinkovine z obarjanjem, pri "top-down" metodah pa zmanjšamo velikost delcev s homogeniziranjem pod visokim tlakom ali z mokrim mletjem v krogličnem mlinu. Pri našem raziskovalnem delu smo uporabili metodo mokrega mletja v krogličnem mlinu in tako pripravili nanokristale glibenklamida, ki je težko topna učinkovina iz skupine sulfonilsečnin. Uporablja se pri zdravljenju sladkorne bolezni tipa II.

Namen tega magistrskega dela je bil razvoj in vrednotenje nanodostavnega sistema z glibenklamidom tj. nanokristalov, ki smo jih izdelali z metodo mokrega mletja v krogličnem mlinu. Na ta način smo želeli povečati hitrost raztapljanja in topnost te težko topne učinkovine, kar je bil naš primarni cilj.

Sprva smo izdelali več različnih formulacij z uporabo dveh različnih stabilizatorjev, Poloxamera 188 in hidroksipropilmetilceluloze (HPMC) ter optimizirali čas mletja. Poloxamer 188 se je izkazal kot boljši stabilizator od HPMC, zato smo ga izbrali za nadaljnje raziskave. Omogočal je nastanek manjših in po velikosti bolj homogenih delcev v primerjavi s HPMC. Velikost nanokristalov, ki smo jih stabilizirali s Poloxamerom 188 je bila manjša od 300 nm že po minimalnem času mletja, v primeru stabilizacije s HPMC pa tako majhnih delcev nismo dosegli niti po 12 ciklih mletja (1 cikel predstavlja 3 min mletja in 15 min premora). Optimalen čas mletja pri uporabi Poloxamera 188 je bil 6 ciklov, zato smo to upoštevali pri pripravi vseh nadaljnjih formulacij.

Nato smo pripravili nanokristale stabilizirane z različno količino izbranega stabilizatorja t.j. Poloxamera 188 in nadalje vrednotili le formulacije, ki so vsebovale 0,1 g, 0,4 g in 0,6 g stabilizatorja na 1 g učinkovine (vzorci NC_{10}, NC_{40} in NC_{60}). S fotonsko korelacijsko spektroskopijo (PCS) smo jim izmerili povprečno velikost delcev in polidisperzni indeks (PI).

Izmerili smo jim tudi zeta-potencial. Kot optimalna količina stabilizatorja se je izkazala 0,4 g na gram glibenklamida (vzorec NC_{40}), saj so bili delci v tem primeru najmanjši (265 nm) in najbolj homogene velikosti (PI 0,2). Nadaljnje vrednotenje smo izvedli tudi z vzorcema NC_{10} (0,1 g Poloxamera 188 na gram glibenklamida) in NC_{60} (0,6 g Poloxamera 188 na gram glibenklamida), saj je bila velikost še vedno zadovoljiva (okrog 305 nm). Zeta-potencial nanokristalov, izmerjen v nasičeni raztopini glibenklamida in stabilizatorja, je bil v vseh primerih okrog –25 mV, kar je odraz negativno nabitih funkcionalnih skupin učinkovine. Naš stabilizator je neionski, kar pomeni, da je sistem sterično stabiliziral, toda prisotnost elektrostatskega odboja zaradi ionizacije same učinkovine je kljub temu prispevala k fizikalni stabilnosti sistema.

Po mletju so nanokristali v obliki nanosuspenzije, ki smo jo liofilizirali z namenom priprave dolgoročno stabilne farmacevtske oblike. Sušenje z zmrzovanjem smo izvedli z in brez uporabe laktoze kot krioprotektanta. Liofilizatov ne glede na prisotnost laktoze nismo mogli zadovoljivo redispergirati v vodnem mediju, kar pomeni, da se je zgradba nanokristalov pri sušenju vsaj delno spremenila. To smo dokazali tudi s pomočjo vrstične elektronske mikroskopije (SEM), kjer smo videli, da so med sušenjem nastali agregati, čeprav so bili posamezni nanometrski delci še vedno vidni. Ker se struktura nanodostavnega sistema ni ohranila, so potrebne nadaljnje raziskave za razvoj in optimizacijo primerne metode sušenja. Pri SEM analizi nismo opazovali zgolj morfoloških značilnosti, temveč tudi velikost nanokristalov v suspenziji, ki je primerljiva velikosti, izmerjeni s PCS.

Izvedli smo diferencialno dinamično kalorimetrijo (DSC) in rentgensko praškovno difrakcijo (XRPD), da bi preverili, ali je glibenklamid ostal kristalen. Tako DSC kot tudi XRPD analiza sta potrdili kristalnost glibenklamida v naših formulacijah. Na DSC krivulji nanokristalov smo zaznali le tališči stabilizatorja in učinkovine, ne pa tudi temperature steklastega prehoda, ki bi nakazovala prisotnost amorfne oblike. S primerjavo XRPD difraktogramov smo prav tako potrdili kristalnost učinkovine, čeprav difraktogram nanokristalov ni povsem ustrezal kristalni obliki glibenklamida. Vrhovi so bili manj izraziti in širši, kar pa je posledica delcev nanometrske velikosti. Z istim namenom smo vzorce analizirali z Ramansko spektroskopijo in izvedli nelinearno optično slikanje, toda rezultati zaradi prisotnosti fluorescentnega stabilizatorja niso bili uporabni. S tekočinsko kromatografijo visoke ločljivosti (HPLC) smo potrdili, da med procesom mletja ni prišlo do kemične razgradnje učinkovine.

Izvedli smo test raztapljanja s farmakopejsko metodo z vesli in tako preverili, ali se je hitrost raztapljanja glibenklamida po vgraditvi v nanokristale izboljšala. Nanokristali v obliki suspenzije so se hitreje raztapljali kot sam glibenklamid in kot fizikalna zmes stabilizatorja in učinkovine, koncentracijski plato je bil dosežen že po 30 s. Tudi sam stabilizator ima vpliv na topnost glibenklamida, zato je bil profil raztapljanja fizikalne zmesi višji kot profil raztapljanja same učinkovine. Vpliv Poloxamera 188 na topnost glibenklamida smo dokazali s proučevanjem topnosti učinkovine v raztopinah z različnimi koncentracijami stabilizatorja in ugotovili, da večje količine Poloxamera 188 močno povečajo topnost naše učinkovine. Test raztapljanja smo izvedli tudi z liofiliziranimi vzorci z in brez dodatka krioprotektanta. Liofilizati brez dodatka laktoze so se raztapljali počasneje kot fizikalna zmes stabilizatorja in učinkovine, kar je posledica nastanka agregatov v procesu sušenja. Z dodatkom laktoze se je raztapljanje izboljšalo, vsaj ob uporabi večjih količin stabilizatorja. Ko smo uporabili najmanjšo proučevano količino Poloxamera 188 (0,1 g na gram učinkovine), se dodatek krioprotektanta ni izkazal za tako učinkovitega.

Intrinzično hitrost raztapljanja smo ugotavljali z uporabo pretočne celice, pri kateri raztapljanje poteka iz konstantne površine. Rezultate meritev lahko povežemo s topnostjo. Topnost nanokristalnih formulacij z večjima količinama stabilizatorja (NC_{40} in NC_{60}) se je izboljšala, pri uporabi manjše količine (NC_{10}) pa ni prišlo do izboljšanja glede na sam glibenklamid. Povečanje topnosti je posledica tako tvorbe majhnih delcev nanometrskih velikosti, kot tudi prisotnosti Poloxamera 188, ki močno vpliva na topnost glibenklamida.

Nanokristale glibenklamida v obliki nanosuspenzij smo shranili zaščiteno pred svetlobo. Vse formulacije (NC_{10}, NC_{40} in NC_{60}) so bile fizikalno in kemično stabilne vsaj 2 meseca, kar smo dokazali z meritvami povprečne velikosti delcev, PI in zeta-potenciala ter s HPLC.

V našem raziskovalnem delu smo z mletjem v krogličnem mlinu uspešno razvili formulacijo s kristalnim glibenklamidom z delci nanometrskih velikosti in tako povečali njegovo topnost in hitrost raztapljanja. Za nadaljnje raziskovalno delo pa ostaja predvsem optimizacija metode sušenja nanokristalov, ki bo zagotavljala nanometrsko velikost delcev tudi po redispergiranju posušenega produkta.

LIST OF USED ABBREVIATIONS

ACN	Acetonitrile
BCS	Biopharmaceutics classification system
CARS	Coherent anti-Stokes Raman scattering
cps	Centipoise
DSC	Differential scanning calorimetry
Eur. Ph.	European Pharmacopoeia
HPH	High pressure homogenization
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methyl cellulose
PCS	Photon correlation spectroscopy
PI	Polydispersity index
PVP	Polyvinylpyrrolidone
rpm	Revolutions per minute
SEM	Scanning electron microscopy
SHG	Second harmonic generation
SLS	Sodium lauryl sulphate
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
T_g	Glass transition temperature
USP	United States Pharmacopoeia
XRPD	X-ray powder diffraction

1. INTRODUCTION

1.1. Structure and stabilization of nanocrystals

Nanocrystals are nanoparticles composed of crystalline drug core covered with a layer of stabilizer. By general definition of nanoparticles, their mean particle size is in nanometer range (1-1000 nm), but most commonly size of pharmaceutical nanocrystals varies from 200 to 500 nm. Nanocrystals as a drug delivery system do not contain any matrix material, just a pure drug and a minimal amount of stabilizing agent that is necessary for a proper stabilization of nanosized particles (1, 2).

Due to their very small size and consequently large surface area nanocrystals have high surface energy, which tends to be minimized by aggregation, precipitation and Ostwald ripening (small particles dissolve and redeposit on larger particles). Stabilizer prevents these phenomena to happen, also during the preparation of nanocrystals, but it is mainly needed for long-term stability (3, 4).

There are two main mechanisms of stabilization: steric and electrostatic (Figure 1). Nonionic surfactants and polymers are used as steric stabilizers due to physical barrier they form on the surface of nanocrystals. Hence, the particles cannot come very close to each other and formation of attractive interactions is impeded. On the other hand, stabilization with ionic surfactants and polymers results in charged surface of nanocrystals and therefore electrostatic repulsion between particles hinders agglomeration process. It is also possible to combine both types of stabilizers to achieve so called electrosteric stabilization, by using two different substances or just by one ionic molecule with long chain length (1).

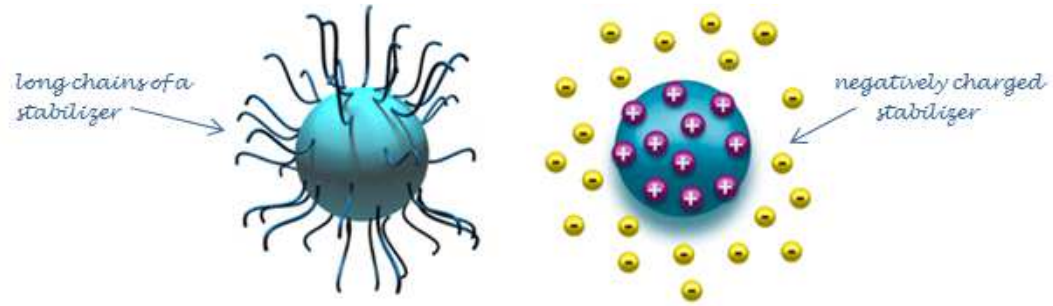

Figure 1: Shematic presentation of steric (left) and electrostatic (right) stabilization (5).

Most commonly used non-ionic surfactants are poloxamers and polysorbate 80. Sodium lauryl sulphate (SLS) is most frequently applied as an ionic surfactant and among steric stabilizers cellulose derivatives, polyvinyl alcohol and povidones are typically used (4).

A choice of a proper stabilizing agent is of crucial importance for achieving a product of satisfactory quality, but unfortunately there is no general formula we could follow. Each drug requires different optimal stabilizer and even preparation technique may influence on that. Therefore, the selection is usually based on empiric procedure, although some stabilizer properties are known to have an impact (1). For sufficient stabilization stabilizing agent should have adequate affinity for the particle surface to form interactions with it. The insoluble drug is generally hydrophobic; therefore, hydrophobicity of stabilizer is an important parameter (1). More hydrophobic agent adsorbs stronger on the particle surface (3). However, stabilizers are usually amphiphilic molecules, where hydrophobic part interacts with hydrophobic drug core and hydrophilic part enables wettability of the particle and later its dissolution in aqueous medium (1). Another important parameter is the viscosity of stabilizer solution. It should not be too viscous, so the diffusion rate is high enough to cover the surface quickly during the preparation process (1, 2). When the stabilization is based on steric repulsion, the polymeric chains need to be long enough to provide sufficient distance between particles. Usually molecular weight of 5.000-25.000 g/mol is recommended (1). The amount of stabilizer used has a big influence on stability as well. It needs to be suitable to cover the whole surface of all particles, but not too high either. Micelles containing dissolved drug can be formed by excess stabilizer (1-3). Micelle formation starts to compete with adsorption of the stabilizer to nanocrystal surface, therefore, less stabilizer and also less drug is available for nanocrystal formation (3). Ostwald ripening is also promoted in suspensions with higher concentrations of stabilizer (1, 6).

1.2. Purpose of nanocrystal formulation

It has been estimated that approximately 40% of all new chemical entities fail development due to their poor solubility in water (7). Nowadays even 70% of all drug candidates are considered as poor water soluble (8). Usual problem of these drugs is their low and unpredictable oral bioavailability, because they tend to be eliminated from the gastrointestinal tract before being completely dissolved and consequently having the opportunity to be absorbed into the circulation (9).

One efficient way to prepare poorly soluble drugs as aqueous dosage forms is to form nanocrystals. By reducing the particle size, the surface area available for drug dissolution increases, what is the main reason for enhanced dissolution rate. According to the Noyes-Whitney equation (Equation *1*) the dissolution rate of the drug is a function of its intrinsic solubility and its surface area, which both increase when nanocrystals are formed and consequently the dissolution rate is increased as well (10).

$$\frac{dm}{dt} = \frac{DA}{L}(Cs - C) \quad \text{[Equation 1]}$$

where

dm/dt = dissolution rate

D = diffusion coefficient

A = surface area of drug particle

L = thickness of diffusion layer

Cs = saturation solubility of the drug

C = concentration in surrounding medium

Solubility, which is in case of micro or macrosized particles a compound-specific constant, is increased, when particles are nanosized what can be explained by Ostwald-Freundlich equation (Equation *2*). This equation applies to materials with mean particle size of less than 2µm (2). The saturation solubility increases with decreasing particle size i.e. smaller particles, especially in nanometer range, have higher saturation concentration on their surface than larger particles (2, 10).

$$\log\left(\frac{Cs}{C\alpha}\right) = \frac{2\sigma V}{2.303RT\rho r} \quad \text{[Equation 2]}$$

where

Cs = saturation solubility of nanosized drug

Cα = solubility of the solid consisting of large particles (bulk material)

σ = interfacial tension

V = molar volume of the particle material

R = gas constant

T = absolute temperature

ρ = density of the solid

r = radius of the particle

Particle size reduction is a nonspecific approach to achieve increased dissolution rate and solubility, applicable for almost all poorly soluble compounds, which are categorized in class II and class IV according to the Biopharmaceutics Classification System (BCS). However, this technique is mostly used for drugs, which belong to BCS class II, as their poor solubility and also poor dissolution rate are the rate-limiting step for their absorption, which can be improved by nanosizing. After dissolution these drugs are well absorbed through the gastrointestinal barrier, because their permeability is high enough, contrary to class IV drugs, which have poor permeability, therefore, improved dissolution alone does not lead to better absorption as well (10-12).

1.3. Different methods of nanocrystal preparation

Drug nanocrystals can be produced by two main technologies: "top-down" and "bottom-up" (Figure 2). In "top-down" methods, such as pearl milling and high pressure homogenization (HPH), the particle size of a coarse powder is decreased. On the other hand, in "bottom-up" methods particles are formed from dissolved drug molecules, usually by precipitation (2). In a novel approach the combination of both technologies is used i.e. "bottom-up" step is followed by a "top-down" technique (12).

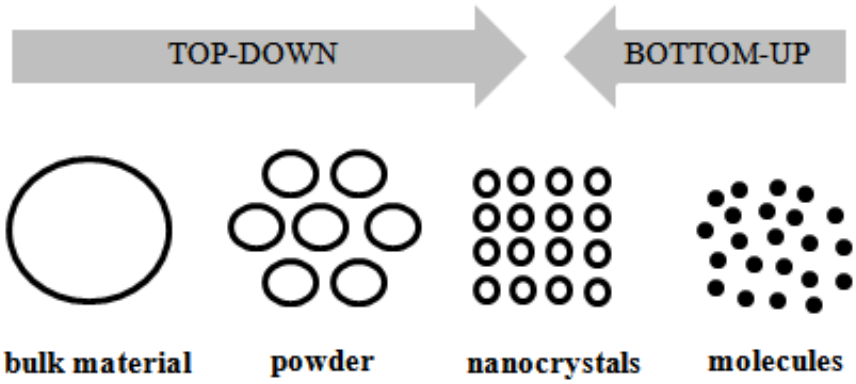

Figure 2: Schematic presentation of "top-down" and "bottom-up" methods for nanocrystal preparation.

1.3.1. "Bottom-up" methods

In precipitation methods poorly water soluble drug is firstly dissolved in an organic solvent and then an anti-solvent (usually water) is added while stirring. As the drug solubility decreases rapidly after addition of an anti-solvent, drug precipitates and nanocrystals are

formed. To determine optimal process parameters including stirring rate, the ratio of solvent and anti-solvent, drug content and temperature can be quite demanding. The method is simple and cost effective when proper conditions are set, but often the problems associated with available solvents appear: poorly water soluble drugs are sometimes insoluble also in many organic solvents, especially if in the method used solvent needs to be miscible with an anti-solvent. Furthermore, the solvent removal can also be demanding, since there should be no solvent residues in the final product. Another drawback of this method is the tendency of particles to grow, therefore, achievement of certain particle size is difficult. Moreover, an amorphous drug can be generated during this process, which brings stability related problems (2).

An alternative way of nanocrystal formulation using a "bottom-up" method is droplet evaporation. The liquid droplets are firstly formed from the drug solution and then solvent evaporation results in nanocrystal formation. Spray-drying is an example where this technology is applied (13).

1.3.2. "Top-down" methods

Among "top-down" methods high pressure homogenization and pearl milling are most frequently applied.

1.3.2.1. High pressure homogenization

Firstly, a macrosuspension of a drug and a stabilizer is prepared in dispersed medium e.g. in water or other non-aqueous media. Subsequently, it is passed through a very thin gap with high velocity inside the homogenizer for several times. The energy generated by cavitation and shear forces is high enough to produce nanosized particles. By modifying the applied pressure and the number of homogenization cycles different particle size can be reached: higher pressure and more cycles usually results in smaller particles with narrower particle size distribution. During the homogenization process high temperature can be generated, what should be taken into account when processing temperature sensitive drugs. When the optimal parameters are achieved, the process has high repeatability with low batch-to-batch variations. Compared to pearl milling, the contamination due to the erosion from the machine is lower (2, 12).

1.3.2.2. Pearl milling

In pearl milling, also called wet-ball milling, micronized drug powder is dispersed in a stabilizer solution and loaded into the milling chamber filled with milling pearls (beads). Subsequently, the drug suspension and pearls are rotated, generating high energy and shear forces which cause size diminution. At the end of milling nanosuspension is produced, which needs to be separated from the beads (1).

Depending on the process parameters different sized nanocrystals can be formed. Drug amount typically varies from 2% to 30% according to total weight of suspension. When using larger amount of drugs, longer milling times are needed and the possibility of aggregation is higher. Milling speed and milling time depend on each other: low milling speed (80-90 rpm) demands longer milling times (1-5 days) and high milling speeds (1.800-4.800 rpm) require shorter milling times (30-60 min) in terms of achieving the same particle size. The latter approach is nowadays more commonly used (1). Amount and size of milling pearls is another very important parameter. Typically, the size of milling pearls is constant and range from 0.5 to 1.0 mm (1), although smaller 0.2 mm beads can also be used (10, 14). Smaller beads are more desirable due to finest particles which can be achieved, but the problem with their separation from the nanosuspension at the end of the milling process occurs. Higher number of pearls results in more contact with drug and consequently to smaller particle formation, but simultaneously increases the loading of the machine and the energy consumption. The main drawback of this method is the product contamination caused by the erosion of milling material, especially pearls. To minimize contamination, shorter milling times and pearls made of highly resistant materials should be used e.g. polystyrene resin, zirconium oxide and glass. The milling temperature also needs to be controlled, mainly when handling with thermolabile drugs with low melting points (1, 2).

During milling two opposite processes are occurring, namely particle size reduction due to fragmentations and particle growth due to interparticle collisions. Which process predominates depends on the parameters used. After certain time a constant average particle size will be reached and additional time and energy input will not further diminish their size. Polymorphic changes or transformation from crystalline to amorphous form can occur during milling process, although this is usually not desirable due to stability related problems associated with amorphous drugs (1).

Since the method is versatile and applicable for almost any active pharmaceutical ingredient, cost-effective, reproducible and easily scaled-up, it is most widely used in the industrial production of nanocrystals. This method is superior over HPH due to smaller particles, which can usually be achieved (1, 12).

In our research work we are going to use this approach for preparation of glibenclamide nanocrystals.

1.3.3. Combined methods

Even though the conventional "top-down" methods described above are widely accepted, they have some disadvantages. Firstly, these methods are energy and time consuming. Secondly, the starting coarse material can clog the equipment. Therefore, new approaches have been developed to overcome these problems. The drug is pre-treated by a "bottom-up" process (usually spray drying or freeze drying) to achieve a suspension of more brittle particles. Due to the modification of the starting material, the following "top-down" process (HPH or milling) is more effective and less homogenization cycles or shorter milling times are needed (12). Also the combination of two "top-down" methods can be utilized, where pre-milled material is further homogenized by HPH (15).

1.3.4. Transformation of nanosuspensions into solid dosage forms

After production nanocrystals are usually in suspension form, therefore drying process is needed before converting them to solid dosage forms. Solid form is preferred over liquid due to the better physical stability and improved patient compliance. Conventional drying methods may be applied, most commonly spray-drying or freeze-drying (4). Freeze-drying, also called lyophilisation is a drying method where liquid sample is firstly frozen and the solvent (ice) is removed by sublimation under low pressure (16). Dry powder may be then used as such or other solid dosage forms, such as capsules or tablets, can be formulated (4).

The critical issue of drying process is usually poor redispersibility of obtained dried material. However, good redispersion should be ensured after addition of medium in order to preserve advantages gained by nanosizing. Unfortunately, aggregation happens quite often during this process, if stabilizer used does not sufficiently protect particles from aggregation. Therefore, cryoprotectants are frequently used (16). Water soluble sugars, such as sucrose, lactose and mannitol, are common cryoprotectants (4, 16). Redispersibility of dried nanocrystals depends

on cryoprotectant concentration and also on parameters used during drying process, e.g. higher freezing rate in freeze-drying process usually results in better redispersibility (16).

1.4. Characterization of nanocrystals

After producing drug nanocrystals it is essential to determine particle´s properties to make sure the required properties have been reached. Among all, the most important are average particle size and particle size distribution, zeta potential, particle shape and morphology, chemical stability, crystallinity, dissolution rate and saturation solubility.

1.4.1. Average particle size and particle size distribution

Particle size is of great importance, as this is the main proof particles are actually in nanosize range. The size also influences on other characteristics, like dissolution rate, saturation solubility and physical stability (2). Distribution of particle sizes in the sample is expressed with polydispersity index (PI). The PI value varies between 0 and 1 and is preferred to be as low as possible, since that signify the particle size distribution is narrow around mean particle size. Samples with PI below 0.2 are defined as monodisperse (17). In more polydisperse samples (higher PI values) Ostwald ripening is more probable. In this phenomenon larger particles grow at the expense of smaller particles leading to an increase in particle size and consequent long-term instability (18).

Both characteristics, average particle size and PI, can be measured by the same instrument based on photon correlation spectroscopy (PCS), also called dynamic light scattering. This is a technique for determination of particle properties typically in the sub-micron region. Sample needs to be homogeneously suspended in a fluid before it is exposed to a laser light, which is than scattered by particles during measurement. PCS detects diffusion rate of nanosized particles, which are moving randomly due to Brownian motion – smaller particles move faster than larger particles (19).

1.4.2. Surface charge

Important for physical stability of colloidal systems is also information about surface charge expressed as zeta potential. Its determination is based on electrophoretic mobility analysed by laser Doppler velocimetry. During the measurement an electrical potential is applied to the sample, which causes the movement of charged particles to the oppositely charged electrode. Their velocity is measured and expressed as electrophoretic mobility, which is then converted

to zeta potential using Henry equation (20). Surface charge needs to be high enough to provide sufficient electrostatic repulsion between particles and consequently the possibility of aggregation is diminished. Preferably its absolute value should be at least 30 mV, if only electrostatic stabilizer is used. If steric stabilization is also involved, stability cannot be estimated based solely on particle zeta potential (1).

1.4.3. Shape and morphology

Shape of particles is an important characteristic, as it is beneficial to know whether the particles are round and homogeneous or they are of indefinite shape and diversely sized. Also morphology of particle surface, if it is flat or rough, can be determined by microscopic analysis. Different types of microscopies can be used to observe particle shape and morphology, transmission electron microscopy (TEM) and more commonly scanning electron microscopy (SEM) can be applied. Samples are microscopically analysed in dry form, therefore potential changes in particle size and shape after water removal are also detected (2).

In SEM measurement an electron beam is focused on the surface of dried sample in a fine 1 nm sized spot. This electron beam is scanned over the material and its interactions with analysed material are recorded on computer, which processes all the information to produce an image. Analysed material needs to be conductive, so the specimen will not gather the charge when exposed to the electrons. Therefore, non-conducting samples are covered by conductive layer prior imaging. It is also important that samples are resistant to electron bombarding and to vacuum conditions used during the measurement (21).

1.4.4. Solid state evaluation

During invasive milling process changes in polymorphic form of a drug can occur. Also an amorphous form can be generated, what is not so probable in pearl milling technique, since the presence of water medium stabilizes the crystalline state and acts as an inhibitor of amorphization (1, 2). On the other hand, in “bottom-up” techniques the formation of amorphous material is more common (13). Amorphous material and instable crystalline polymorphs are not desired, even though they might have better solubility characteristics. Stability related problems during storage may occur, as amorphous particles tend to transform to stable crystalline form very fast and consequently physical properties are changed as well (2). To evaluate crystallinity of produced nanocrystals different methods can be used, most commonly differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD) are performed.

DSC is a thermal method, where the temperature of the sample and the inert reference are measured at the same time while exposed to heating. The difference in energy (heat flow) is recorded and expressed as a function of temperature in a thermogram, where different thermal processes can be observed. Endothermic peaks (energy is absorbed) are characteristic for melting, phase transition or solvent loss, while exothermic peak (energy is released) represents for instance crystallisation. If the sample is crystalline, endothermic peak will be present due to melting and in case of amorphous material baseline shift due to glass transition temperature (T_g) and exothermic peak due to crystallisation can be observed (22). In nanosized materials melting temperature is usually decreased and broadening of melting peak appears (23).

During XRPD measurement sample is exposed to x-rays, which are scattered from atoms in the substance producing a diffraction pattern, which contains information about the atomic arrangement in the crystal. Each crystal structure has a unique diffraction pattern, typically with many sharp and well-defined peaks. On the other hand, amorphous material does not have an ordered structure to produce diffraction pattern, so the diffractogram obtained consists of broad indefinite peaks (22). Even when analysing crystalline samples, broadening of the peaks may occur due to presence of nanosized particles and subsequently results can be misinterpreted as having an amorphous material, even though it is crystalline. This needs to be taken into account when analysing material in nano range (24).

Raman spectroscopy can also be performed to determine changes in solid state. During the measurement monochromatic excitation source (laser) strikes the material and interacts with its molecular vibrations, what can lead to Raman scattering. The Raman signal spectrum is obtained by measuring the intensity of scattered photons as a function of the frequency. It is characteristic for each substance. A competitive phenomenon, the fluorescence, can occur during the measurement. When the sample is irradiated by a laser, it can absorb some energy and reemits it as fluorescence. Even if the sample is just a little bit fluorescenting, the signal is still stronger than Raman scattering and can easily prevail over weak Raman signal, which can be seen as a broad fluorescence bend in the spectrum. The effect of the fluorescence can be removed by reprocessing the process parameters (25, 26).

Another quite novel technique can be performed to evaluate crystalline state, namely non-linear optical imaging. In this method a sample is irradiated by laser with two or more photons interacting with each other and forming new photon of another wavelength which is then

detected. Main advantages of non-linear imaging over linear are better chemical selectivity and faster analysis, which enables real time monitoring. Even though non-linear optical imaging has not been widely used in pharmaceutical applications, it can be useful also in this field. It includes different techniques, but only the second harmonic generation (SHG) and coherent anti-Stokes Raman scattering (CARS) will be explained briefly (27), since other techniques will not be used in our research.

Energy level diagrams of different non-linear optical techniques are presented in Figure 3. In SHG two lasers interact with virtual state of the substance emitting a photon of another wavelength. In CARS the frequency difference between the pump photon (ω_p) and Stokes photon (ω_s) matches a vibrational resonance in the substance and increase its polarisation. When third photon (ω_{pr}) probes the polarisation, a CARS signal is generated at higher frequency. In Raman spectroscopy (which is not part of non-linear optical techniques) there is just one laser interacting with the sample emitting Stokes Raman scattering at lower frequency (27).

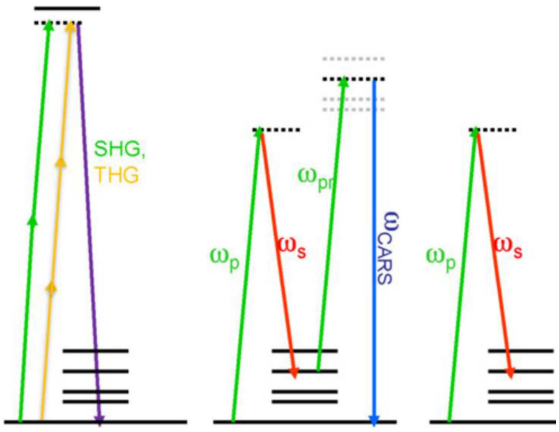

Figure 3: Energy level diagrams of different non-linear optical techniques. Left: second-harmonic generation (SHG); middle: coherent anti-Stokes Raman scattering (CARS); right: Stokes Raman scattering (27).

Due to its high specificity CARS is used to determine the drug and its distribution in the sample. SHG signal can be used to analyse structural information, as it can distinguish between crystalline and amorphous material. Substances with inversion symmetry and amorphous material cannot be detected, whereas crystalline form can generate second harmonic signal and is thus seen on the screen (27).

1.4.5. Saturation solubility and dissolution rate

It is important to determine saturation solubility and dissolution rate, as these two parameters increase when nanosized particles are formed. Since the improvement of drug dissolution is usually the aim of nanosizing process, the research can be evaluated based on the outcome of this determination. On top of that, when dissolution behaviour of material is known, its *in vivo* performance can be easier predicted (2).

Different methods described in pharmacopoeia can be applied for determination of dissolution rate, most commonly paddle or basket method are used (28), where dissolution occurs from whole particle surface. Because the dissolution of nanosized particles is so fast, the information about the beginning of the dissolution process is often lost in these methods. Therefore, it is convenient to determine also intrinsic dissolution rate i.e. dissolution of pure substance over a constant surface area. In intrinsic dissolution method only one side of the compressed drug i.e. tablet is exposed to the medium and thus the beginning of the dissolution process can be followed as well (29, 30). There is a method described in European Pharmacopoeia (Eur. Ph.) for determination of intrinsic dissolution rate (28), but channel flow method (flow-through cell) has also been established to correlate extremely well with pharmacopoeian method. This flow-through cell is not the same as flow-through cell explained in Eur. Ph. for dissolution test of solid dosage forms (28), since the dissolution is happening from a flat and constant surface, unlike in pharmacopoeian method, where a tablet can disintegrate and the dissolution is thus not occurring from a constant surface anymore (29). Evaluation of intrinsic dissolution rate can be considered as a technique to classify solubility, since the relationship between these two properties has been found (29, 30). In conventional shake flask method more time and material is needed to obtain saturated solution and then to determine solubility, which is improved in case of nanosized particles (30).

To determine drug concentration in a sample, different methods may be used. High performance liquid chromatography (HPLC) is very commonly used, as different compounds are firstly separated based on their hydrophobicity and then their concentration is detected, usually by UV detector. In reverse phase HPLC more hydrophobic molecules are longer retained on hydrophobic stationary phase and therefore elute later from the column. In the chromatogram each component is characterized by a separate peak which can be quantified and any degradation products present in the sample can also be observed (31). UV spectrophotometer can also be used for determination of drug concentration, but its specificity is lower, as there is no separation among different compounds in the sample, unlike in HPLC

method. Concentration is recorded at defined prior analysis selected wavelength, which should be specific for the drug (32).

1.5. Glibenclamide and its nanocrystals

Glibenclamide (Figure 4) is a drug belonging to sulfonylurea group used in the treatment of non-insulin dependent diabetes mellitus (diabetes type II). Its hypoglycemic activity is due to the stimulation of β cells in pancreas, which consequently release insulin. According to the BCS it is classified as a class II drug with typically poor water solubility (less than 4 mg/L (33)) and quite high permeability, therefore, glibenclamide is an ideal drug for preparation of nanocrystals to improve its dissolution rate and consequently its bioavailability (10, 34). Glibenclamide has a melting point of 173-175°C and pKa value of 5.1 (35).

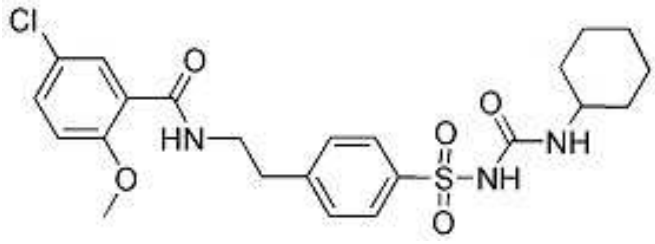

Figure 4: Chemical structure of glibenclamide (35).

Glibenclamide nanocrystals have been previously prepared using different techniques: "bottom-up" (36-38), "top-down" (14) and also the combination of both methods (10). Different stabilizers have been used in research, but usually the combination of two proved to be the most effective (14, 36-38).

Precipitation as a "bottom-up" method for preparation of glibenclamide nanocrystals was applied by various researchers (36-38). Combination of two non-ionic surfactants commercially available as Solutol HS-15 and Cremophor RH40 was used to prepare microcrystals with average size around 2 µm (36). In another research nanocrystals with particle size below 300 nm were formulated with hydroxypropyl methyl cellulose (HPMC) in combination with polyvinylpyrrolidone (PVP K-30) as stabilizers (37). In a precipitation method a mixture of surfactants Poloxamer 188 and polymer PVP was also employed, which proved to be the best in terms of the smallest particles achieved. In the same study different stabilizers were tested separately and Poloxamer 188 resulted in the finest nanocrystals with the average size around 200 nm (38).

Among “top-down” methods pearl milling was used to manufacture glibenclamide nanocrystals with HPMC and SLS as stabilizers (14).

Quite novel approach is a combined method, where freeze-drying process is followed by “top-down” step, namely milling or high pressure homogenization. In this investigation sodium docusate was used as an ionic stabilizer (10).

According to the literature review, Poloxamer 188 and HPMC have been chosen for initial screening in our research. Poloxamer 188 proved to be the best among many investigated stabilizers when precipitation method had been used (38) and HPMC was investigated in two different studies (14, 37).

1.5.1. Poloxamer 188

Poloxamer 188 is a nonionic block copolymer of ethylene oxide and propylene oxide, as presented in Figure 5. It is a solid, freely soluble in water and in ethanol with a melting point between 52 and 57°C. It is nontoxic and nonirritant material; therefore, it has many applications in pharmaceutical formulation, mainly as an emulsifying and solubilizing agent (39).

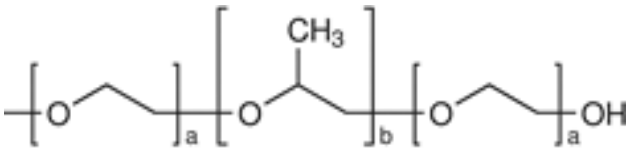

Figure 5: Chemical structure of Poloxamer 188, where block a has a value of 80 and block b 27 (40).

Poloxamer 188 is widely used also as a stabilizing agent for nanocrystals, since it is nonionic surfactant which forms hydrophobic interactions with particle surface and stabilizes the system mainly due to formation of steric barriers (2).

2. OBJECTIVES

The main purpose of our research work is to develop and evaluate nanocrystalline formulation of glibenclamide by pearl milling technique in order to improve dissolution of this poorly water-soluble drug and consequently its bioavailability.

Firstly, a type of a stabilizer and its concentration, as well as milling time, will be optimized. The evaluation of initially produced nanocrystals will be based on particle size, particle size distribution and zeta potential measurements. After selection of optimal formulation with smallest and most homogeneous particle size, further characterization of produced nanocrystals will follow.

The nanocrystal formulations will be freeze-dried with and without addition of cryoprotectant to prepare dry powder. Particle shape and morphology of dried samples will be observed by scanning electron microscopy (SEM). The preservation of crystallinity after milling process will be checked on freeze-dried product by different techniques: differential scanning calorimetry (DSC), x-ray powder diffraction (XRPD) and Raman spectroscopy. Quite novel method named non-linear optical imaging will be applied for this purpose as well. To evaluate possible presence of chemical degradation during the milling process HPLC analysis will also be carried out.

The most important aim of our research is the improvement of glibenclamide dissolution rate, which will be evaluated in dissolution test performed with pharmacopoeian paddle method. Intrinsic dissolution will be determined by flow-through cell set-up to evaluate the improvement in solubility due to formation of nanosized particles.

Produced nanocrystals will be stored at room conditions. Their stability will be investigated throughout 2 months period by average particle size, particle size distribution and zeta potential determinations.

3. MATERIALS AND METHODS

3.1. Materials

A drug used in our study i.e. glibenclamide was purchased from Berlin Chemie (Berlin, Germany). Two different polymers were tested as potential stabilizers: Poloxamer 188 (Lutrol F68, BASF, Ludwigshafen, Germany) and HPMC (Methocel E5 premium LV EP, The Dow chemical company, Midland, Michigan, USA).

Lactose monohydrate (Pharmatose 200M, DMV International, Veghel, Netherlands) was used as a cryoprotectant during freeze-drying. Ethanol (Aa quality, 99.5%, Altia, Finland) was used as a solvent in HPLC analysis.

For dissolution test 0.05 M phosphate buffer with pH 7.5 was used, which had been prepared according to the United States Pharmacopoeia (USP) (41). Firstly, the 0.2 M NaOH solution and 0.2 M KH_2PO_4 solution were prepared separately. 8 g of NaOH was weighed into volumetric flask and dissolved in 1 l of Milli-Q water. For preparation of 0.2 M KH_2PO_4 27.22 g of KH_2PO_4 (Sigma-Aldrich, Steinheim, Germany) was dissolved in 1 l of Milli-Q water. To obtain 1 l of 0.05 M phosphate buffer 250 ml of 0.2 M KH_2PO_4 solution and 205 ml of 0.2 M NaOH solution were mixed together and diluted with Milli-Q water to volume specified. pH was adjusted to 7.5 with 0.2 M NaOH, using pH-meter (pH/mV-Meter Fieldlab, Schott, Mainz, Germany).

For flow-through cell measurement boric buffer with pH 9.0 was prepared in accordance with USP (41). Firstly, 0.2 M NaOH solution was prepared by dissolving 8 g of NaOH in 1 l of Milli-Q water, whereas 0.2 M solution of boric acid and potassium chloride was prepared by adding 12.37 g of boric acid (Sigma-Aldrich, USA) and 14.91 g of KCl (Sigma-Aldrich, Germany) into 1 l of Milli-Q water. 250 ml of 0.2 M boric acid and KCl solution and 104 ml of 0.2 M NaOH solution was mixed and then diluted with Milli-Q water to the total volume of 1 l. pH was adjusted to 9.0 with 0.2 M NaOH or 0.2 M boric acid and KCl solution.

3.2. Preparation of glibenclamide nanocrystals

3.2.1. Pearl milling

Nanocrystals were prepared using wet-ball milling technique. The procedure was the same in all experiments. Firstly, the suspension of glibenclamide in aqueous solution of stabilizer was prepared. One day before milling, a solution with suitable concentration of Poloxamer 188 or HPMC had been prepared by dissolving stabilizer in 3 ml of Milli-Q water and then it was put in the fridge for at least 12 h so that it dissolved completely. Just prior milling 1 g of glibenclamide was weighed in 2 ml of Milli-Q water and added to the stabilizer solution prepared previous day. Whole 5 ml of aqueous drug suspension was put in the grinding bowl together with 30.0 grams of zirconium oxide pearls (Fritsch GmbH, Germany) having a diameter of 1 mm (Figure 6). Before covering the bowl with the lid, a flat seal with small groove was added in between to release high pressure created during the milling process.

Figure 6: Grinding bowl: (a) closed and (b) opened and filled with milling pearls.

Planetary mill Pulverisette 7 premium line (Fritsch GmbH, Germany) was used to produce nanosuspensions (Figure 7). Milling was carried out at maximum speed of 1100 rpm in 6 cycles. Each milling cycle included 3 min of milling and 15 min of break without change of the direction of rotation between the cycles. Quite long break was necessary to prevent the vessel and the sample from overheating due to high energy generated during milling. After every second cycle the vessel was taken out of the machine for 25 min to be cooled down below 30°C. Finally, the nanosuspension was separated from the pearls by a pipet and transferred to a vial, which was sealed with parafilm and stored protected from light for further analyses.

Figure 7: Planetary mill used for pearl milling in our research.

3.2.2. Selection of stabilizer and optimal milling time

Two different stabilizers were tested initially: Poloxamer 188 and HPMC. Preliminary experiment with each of them separately was performed with 0.4 g of stabilizer per 5 ml of dispersion. Different batches with each stabilizer were prepared according to the previously described procedure to see how particle size changes with increase in milling time. Total milling time, expressed as a number of milling cycles, was 10 in case of Poloxamer 188 and 12 when HPMC was used as a stabilizer. Samples were taken after every second milling cycle, particle size and polydispersity index were analysed with PCS straightaway.

3.2.3. Selection of optimal stabilizer concentration

Nanocrystals stabilized with different amounts of selected stabilizer (Poloxamer 188) were manufactured according to the procedure described in section 3.2.1 to see the effect of stabilizer concentration on particle size and polydispersity. All the samples contained the same amount of the drug and water, only the amount of used stabilizer was changed in different formulation compositions (Table I). Average particle size, particle size distribution and zeta potential were measured straightaway after nanocrystals production. Based on the results obtained by these analyses further characterisation was performed only with nanocrystals stabilized with 0.1, 0.4 and 0.6 g of stabilizer per gram of drug (NC_{10}, NC_{40} and NC_{60}).

Table I: Composition of formulations used for preparation of glibenclamide nanocrystals.

Sample	$m_{glib.}$ (g)	$m_{stab.}$ (g)	V_{medium} (ml)
NC_{10}	1	**0.1**	5
NC_{20}	1	**0.2**	5
NC_{30}	1	**0.3**	5
NC_{40}	1	**0.4**	5
NC_{60}	1	**0.6**	5

In some analyses produced glibenclamide nanocrystals were compared to pure glibenclamide or to the physical mixtures of drug and stabilizer, which had been prepared by mixing 1 g of glibenclamide and the corresponding amount of the stabilizer (0.1, 0.4 or 0.6 g) by the rule of geometric mixing.

3.2.4. Freeze-drying

Final product obtained after milling is a liquid nanosuspension, but for many analyses dry sample is required. Therefore, the necessary step in our research was freeze-drying of glibenclamide nanosuspensions (NC_{10}, NC_{40}, NC_{60}). After production of nanocrystals half of the sample was stored as a suspension and the other half (2.5 ml) was put on Petri dish in order to be dried. Firstly, the sample was frozen in a freezer at -20°C for 1.5 h. Then it was transferred to freeze-dryer (HETO LyoPro 3000 Freeze Dryer, Heto-Holten A/S, Allerød, Dennmark), where it was dried at <0.01 hPa and -53°C for 3 days.

The freeze-drying was performed in absence and presence of cryoprotectant. Lactose, which is commonly used as a cryoprotectant in freeze-drying process (16), was used in our research. Firstly, lactose solution was prepared by dissolving 1 g of lactose in 10 ml of Milli-Q water. 0.5 ml of lactose solution was added to 2.5 ml of nanosuspension prior freeze-drying. In this way, weight to weight ratio between drug and cryoprotectant was 10:1.

3.2.5. Solubility test

Three saturated solutions of glibenclamide in water were prepared containing different amounts of the stabilizer. The excess amount of the drug was added to each solution of the stabilizer (2%, 8% and 12% (w/v)), then stirred for 24 hours and filtered through 0.45 µm membrane filter to remove undissolved drug. 0.5 ml of ethanol was added to 0.5 ml of filtered

sample and analysed with HPLC. Saturated solution of glibenclamide in pure water was also prepared and analysed.

3.3. Characterization of glibenclamide nanocrystals

3.3.1. Particle size analysis

Particle size and polydispersity index (PI) of nanosuspensions were measured using Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The measurements were carried out on the same day as milling. Prior to the analysis, all the nanosuspensions needed to be diluted with saturated glibenclamide solution, which had been prepared one day before the analysis by addition of an excess amount of drug to aqueous stabilizer solution (0.1 g of stabilizer in 100 ml of Milli-Q water). After mixing for approximately 12 h, it was filtered through 0.45 µm membrane filter. Every week new saturated solution was prepared. The purpose of using saturated solution and not just water was to ensure that the drug in nanocrystals would not dissolve due to dilution during the analysis. All the samples were diluted approximately 5.000 times. Each sample was analysed three times at 25°C.

Particle size of dried samples was also determined by PCS after redispersion. Freeze-dried sample was redispersed in saturated glibenclamide solution prepared as previously described. Samples were then mixed well on vortex. Due to poor redispersibility, samples were sonicated for 10 min using an ultrasonic bath prior PCS analyses.

3.3.2. Zeta potential measurement

Zeta potential was measured with Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Sample preparation was the same as in particle size measurements. Each sample was analysed three times at 25°C in a cuvette with two electrodes on both sides. Smoluchowski approximation was used to convert electrophoretic mobility to zeta potential.

3.3.3. Scanning electron microscopy (SEM)

The particle size and surface morphology of the samples were characterized by a FEI Quanta 250 FEG (FEI Inc., Eindhoven, Netherlands) scanning electron microscope equipped with Everhart-Thornley detector. Nanosuspensions were diluted 20 times with saturated glibenclamide solution (its preparation is described in section 3.3.1). A 9 µl drop was put on a holder and let to dry at room conditions one day prior the analysis, whereas the dried samples were put on holders just before the measurement. All the samples were fixed with a double-

sided conductive carbon tape on top of aluminium studs and coated with 5 nm thick layer of platinum. Each sample was analysed at acceleration voltage of 5 kV.

3.3.4. Differential scanning calorimetry (DSC)

The samples were analysed using Mettler DSC 823e (Mettler-Toledo AG, PTD 2007-2555, Greifensee, Switzerland). Approximately 5 mg of dried sample was weighed into an aluminium pan, compressed by a metal rod to get flat surface, assuring more stable enthalpy. The pan was covered with a lid in which two holes had been made, hence gas residuals could evaporate during the measurement. Each sample was stabilized at 0°C for 5 min and then heated to 200°C with a heating rate of 10°C/min using 50 ml/min N_2 flow. The results were analysed using STAR^e software version 9.0 also provided by Mettler-Toledo, Switzerland. Pure glibenclamide, pure Poloxamer 188, two nanocrystal samples (NC_{10} and NC_{40}) and two corresponding physical mixtures of drug and stabilizer (0.1 g and 0.4 g of stabilizer mixed with 1 g of drug) were examined. A single measurement was performed for each sample.

3.3.5. X-ray powder diffraction (XRPD)

XRPD measurement was implemented to analyse the crystallinity of the samples using the Bruker D8 Advance system (Bruker AXC GmbH, Karlsruhe, Germany) with Cu Kα radiation of λ=1.542 Å (40 kV and 40 mA). Some powder of each sample was put into aluminium sample holder and gently pressed with spatula to ensure flat surface. Each sample was scanned from diffraction angle (2θ) of 5° to 40° with a step size of 0.05° and measured for 1 s on each position. Pure Poloxamer 188, two nanocrystal samples (NC_{40} and NC_{60}) and two corresponding physical mixtures of drug and stabilizer (0.4 g and 0.6 g mixed with 1 g of drug) were analysed, while crystalline and amorphous glibenclamide spectrums were obtained from previous studies. A single measurement was performed for each sample.

3.3.6. Raman spectroscopy

Raman spectroscopy was performed using the PhAT system (Kaiser Optical Systems, Ann Arbor, PTD 2008-1617, MI, USA) equipped with a 785 nm laser, a probe which consisted of an array of 50 optical fibers and an air-cooled charge-coupled device detector. The sampling spot size of this system was 6 mm in diameter and the size of the area illuminated was 28.3 mm^2. Each sample protected from light was exposed to the laser for 5 s and scanned 5-times to obtain the average spectrum. Samples were analysed in tablet form (prepared as described later in section 3.3.10) during flow-through cell measurement in 5 min intervals in order to

see if crystallinity of drug is changing during dissolution. Spectrums were obtained by HoloGRAMS^{TM} 4.1 software (Kaiser Optical Systems).

3.3.7. Non-linear optical imaging

CARS and SHG imaging were performed with a Leica TCS SP8 CARS instrument which contains a Leica DMI 6000 inverted microscope and two forward CARS and two EPI CARS detectors. CARS signal has a detection range of 560-750 nm and SHG signal 380-550 nm. Samples containing glibenclamide were irradiated by 2 lasers (wavelength of 817 nm and 1064 nm). The objective with 63-times magnification was used with glycerol immersion on the surface of the sample holder that was being imaged. Freeze-dried samples containing different amounts of stabilizer (NC_{10}, NC_{40} and NC_{60}) and pure stabilizer were analysed, whereas pure glibenclamide had been recorded by previous researchers.

3.3.8. HPLC analysis

To determine the concentration and presence of any degradation products of glibenclamide in different samples HPLC analysis was performed using HPLC instrument Agilent 1100 series (Agilent Technologies, Germany) with an UV detector. The column used for separation was Gemini 3 µm NX-C18 110Å, LC column 100x4.6 mm (Phenomenex, Denmark). Details of the method are presented in Table II. The retention time of glibenclamide at conditions specified was around 3 min.

Table II: Conditions of HPLC method used for determination of glibenclamide.

Parameter	HPLC condition
Mobile phase	ACN:TFA(0.1% (v/v); pH 2) = 60:40
Injection volume	20 µL
Flow rate	1.0 ml/min
Detection wavelength	230 nm
Column temperature	25°C

Preparation of mobile phase

Mobile phase used during the measurement consisted of acetonitrile (ACN, HiPerSolv CHROMANORN, VWR Prolabo, Fontanay-Sous-Bois, France) and 0.1% (v/v) trifluoroacetic acid with pH 2. The 0.1% (v/v) solution of TFA was prepared by addition of 1 ml of TFA (Sigma-Aldrich, Germany) to 1 l of Milli-Q water. pH was measured with pH-meter

(Fieldlab, Germany) and adjusted to pH 2.0 with 1 M HCl or 1 M NaOH, if necessary. The solution was filtered through 0.45 µm membrane filter prior use.

Evaluation of glibenclamide stability in pearl milling process

The drug content and possible degradation during the milling process was evaluated on two fresh samples of nanosuspensions (NC_{10} and NC_{40}). 15 ml of ethanol was added to approximately 1.5 mg of each nanosuspension and stirred on magnetic stirrer for 2 h to assure complete particle dissolution. Then 0.5 ml of Milli-Q water was added to 0.5 ml of prepared sample and analysed with HPLC using the conditions described in Table II.

3.3.9. Dissolution test

The effect of milling on the dissolution rate of glibenclamide was determined by the paddle method (Sotax, Basel, Switzerland) described in Eur. Ph. (28). The test conditions used were chosen according to the Food and Drug Administration recommendations for micronized drug: 900 ml of 0.05 M phosphate buffer solution with a pH 7.5 and the rotation speed of 50 rpm (42). 0.05 M phosphate buffer with pH 7.5 was prepared according to the procedure described in section 3.1. The water bath maintained the temperature of the medium at 37 ± 0.5°C. Samples of 3 ml were withdrawn at 0.5, 1, 1.5, 2, 5, 10, 15, 30, 60 min time points and replaced with fresh medium to maintain the constant volume of dissolution medium. After sampling all samples were stored in the fridge protected from light. Just before HPLC analysis, 0.5 ml of ethanol was added to 0.5 ml of each sample, mixed well and analysed according to the HPLC method described in section 3.3.8.

Nanocrystals in a suspension (NC_{10}, NC_{40} and NC_{60}) and corresponding freeze-dried nanocrystals with and without lactose were tested. In this way three different amounts of the stabilizer were evaluated in each group. Pure glibenclamide and a physical mixture containing 0.6 g of stabilizer and 1 g of glibenclamide were also analysed. Each sample was tested twice. The amount of the sample used for the dissolution test contained 5 mg of the drug. The exact amount of glibenclamide in freeze-dried samples was determined by HPLC, as described below.

The same amount of the sample that had been used for dissolution test (theoretically containing 5 mg of drug) was added to 50 ml of ethanol and mixed on magnetic stirrer for 3 h to assure complete dissolution. 0.5 ml of water was then added to 0.5 ml of that solution and analysed with HPLC. The percentage of the drug dissolved in each time point was calculated

based on the total amount determined in these separate analyses of freeze-dried samples. For nanosuspension samples (NC_{10}, NC_{40}, NC_{60}) theoretical value i.e. 5 mg was taken as a total amount of the drug for calculating the percentage of the drug dissolved.

3.3.10. Evaluation of intrinsic dissolution rate

Intrinsic dissolution rate was measured with flow-through cell setup. To ensure flat surface necessary for this assay, a tablet had been firstly prepared using the Specac Hydraulic Press Model 15.011 (Specac, Kent, UK) equipped with a 13 mm diameter flat faced punch. A tablet was prepared from approximately 150 mg of powdered sample compressed at 0.5 ton with a dwell time of 30 s. The samples evaluated in this test include freeze-dried nanocrystals containing three different amounts of stabilizers (freeze-dried NC_{10}, NC_{40} and NC_{60}). Corresponding physical mixtures of drug and stabilizer and pure glibenclamide were also analysed. After compression the tablet was inserted inside the flow-through cell in a way to be exposed to the medium only from one side during the measurement.

Dissolution medium was 900 ml of boric buffer with pH of 9.0, prepared according to the procedure described in section 3.1. Its temperature was maintained at 37 ± 0.5°C by water bath. It was stirred with a paddle at a rate of 50 rpm. During the procedure the medium was circulating throughout the system, which consisted of the buffer reservoir, the UV/VIS spectrophotometer, the pump and the flow-through cell with a sample tablet (Figure 8).

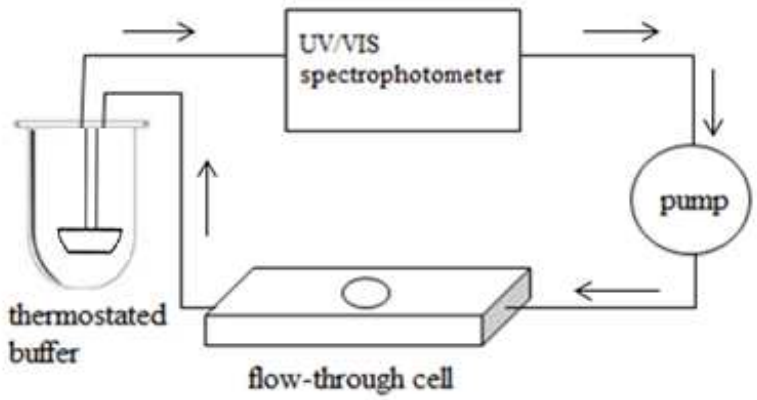

Figure 8: Scheme of flow-through cell system.

The pump was generating the medium flow rate of 4 ml/min. The detection of the drug dissolved was carried out by UV/VIS spectrophotometer (UV-1600 PC Spectrophotometer, VWR, China) connected to a computer (M. Wave Professional software, version 1.0, provided by VWR) which enabled simultaneous measurement of sample absorbance at predetermined

time intervals and drawing of a dissolution profile. In the test, which was performed for 1 h, the sample absorbance was recorded every minute at the wavelength of 230 nm. Before flow-through cell analysis, the whole absorbance spectrum of a drug solution and a solution of drug with stabilizer had been scanned from 200 nm to 500 nm in order to assure that the response measured at 230 nm resulted from the drug and not from the stabilizer.

3.3.11. Evaluation of physical stability of nanocrystal dispersions

Physical stability testing was performed throughout 2 months period. Particle size, PI and zeta potential of nanosuspensions NC_{10}, NC_{40} and NC_{60} were determined 1, 2, 4 and 8 weeks after production. Average results were calculated from measurements of two different batches containing the same amount of stabilizer. Samples were stored in vials sealed with parafilm at room temperature and protected from light.

4. RESULTS AND DISCUSSION

4.1. Formulation and milling optimization

4.1.1. Selection of stabilizer and optimal milling time

A choice of a proper stabilizing agent is of great importance for the stability of prepared nanocrystals and this is the reason we initially focused our study on selection of a suitable stabilizer. In the preliminary experiment two different stabilizers (Poloxamer 188 and HPMC) were evaluated. 0.4 g of each stabilizer was used, as this is, based on literature data, the most reasonable concentration. This amount has been confirmed as the most optimal also for other stabilizer used in previous study (14). Results of particle size and PI measurements, which were performed straight after milling, are represented in Table III. Based on the results Poloxamer 188 was chosen for further studies due to smaller and more homogenous particles achieved compared to HPMC stabilized dispersions. Optimal milling time was shown to be 6 milling cycles, when Poloxamer 188 was used as a stabilizer.

Table III: Particle size (d) and polydispersity index (PI) of glibenclamide nanosuspensions stabilized with 0.4 g of stabilizer (Poloxamer 188 or HPMC) per gram of drug. Results are presented as average ± standard deviation of three measurements.

Number of	**Poloxamer 188**		**HPMC**	
milling cycles	d (nm)	PI	d (nm)	PI
2	295.6 ± 1.2	0.235 ± 0.016	475.4 ±1.3	0.329 ±0.019
4	286.5 ± 2.8	0.260 ± 0.008	464.8 ±4.4	0.336 ± 0.015
6	260.6 ± 2.5	0.176 ± 0.020	446.8 ± 4.9	0.266 ± 0.021
8	271.8 ± 1.7	0.219 ± 0.013	411.9 ± 3.2	0.236 ± 0.029
10	293.1 ± 3.6	0.223 ± 0.011	378.6 ± 7.9	0.214 ± 0.008
12	/	/	305.8 ± 2.5	0.252 ± 0.013

When using HPMC as a stabilizer, an expected trend was observed: longer milling times resulted in smaller particles. But even after 12 milling cycles, average particle size and PI were higher than in all samples of nanocrystals stabilized with Poloxamer 188. Shorter milling times are desired when optimising the process due to lower probability of drug degradation and lower energy consumption. On top of that, the suspensions containing HPMC had very high viscosity, making milling less effective due to slow diffusion of stabilizer molecules in the process of covering new particle surfaces formed during milling. This can also be a reason why it took more time to reach smaller particles when HPMC was used. High viscosity also makes the suspension separation from the milling pearls more demanding. Quite significant amount of suspension adhered to the pearls and consequently less final product was produced. Beside smaller and more homogeneous particles achieved with Poloxamer 188, all of the above are reasons why HPMC was not chosen as an optimal stabilizer for further experiments.

However, HPMC stabilized nanocrystals were not studied further, but the stability test of these HPMC stabilized nanocrystals was performed anyway. Nanocrystals prepared with 12 cycles of milling were stored for 8 weeks protected from light. During stability testing average particle size and also PI increased from approximately 300 to 400 nm and from 0.25 to 0.45, respectively. Zeta potential of particles was much lower, around – 10 mV, compared to around – 25 mV for nanocrystals stabilized with Poloxamer 188. All these findings indicate that stabilization with HPMC was not successful and confirming that our choice of stabilizing agent was correct.

In case of Poloxamer 188 the best result was obtained when 6 milling cycles were employed. The minimal particle size was achieved and additional milling did not further diminish the particles, contrary, they even got slightly larger again. According to the literature this phenomenon is quite common in the milling process (1).

4.1.2. Selection of optimal stabilizer concentration

Different amounts of Poloxamer 188 as a stabilizer were tested in order to select the optimal composition of the formulation. The minimal amount of stabilizing agent, which still adequately stabilizes the system, is desired. Prepared nanocrystals were evaluated according to the results obtained by PCS measurement. All the results are presented in Table IV.

Table IV: Particle size (d), polydispersity index (PI) and zeta potential (ZP) of nanocrystals produced with different amounts of Poloxamer 188 as a stabilizer. Results are presented as average ± standard deviation of three measurements.

Sample	**d** (nm)	**PI**	**ZP** (mV)
NC_{10}	303.3 ± 1.3	0.225 ± 0.015	-28.8 ± 0.5
NC_{20}	264.5 ± 2.1	0.191 ± 0.019	-25.5 ± 0.7
NC_{30}	273.2 ± 1.9	0.202 ± 0.010	-25.3 ± 0.4
NC_{40}	264.0 ± 1.0	0.197 ± 0.002	-23.9 ± 0.3
NC_{60}	306.0 ± 3.8	0.271 ± 0.008	-22.8 ± 0.5

There was no significant difference in particle size and PI between all prepared samples. Average particle size varied between 260 and 280 nm, whereas PI was around 0.2 in most nanocrystal samples. In sample NC_{10}, where the smallest amount of stabilizer was used, average particle size was a bit above 300 nm, suggesting this amount of stabilizer did not stabilize the system as much as higher amounts of Poloxamer 188 used in other nanocrystal samples. When 0.6 g of stabilizer was used per gram of drug (sample NC_{60}) average particle size was greater as well and particles were also more polydisperse. An explanation for this phenomenon can be found in overreaching the sufficient amount of the stabilizer necessary for adequate stabilization. Micelles containing dissolved drug can be formed from the excess stabilizer. Micelle formation starts to compete with adsorption of the stabilizer to nanocrystal surface; therefore, less stabilizer is available for adsorption on newly formed surfaces of nanocrystals resulting in insufficiently stabilized system (1-3). Particles may be larger also because of thicker stabilizing layer formed around the solid drug core. Altogether, additional amount of stabilizer apparently negatively affects the effectiveness of the particle size reduction.

Zeta potential was quite comparable among all samples. Its values were between – 22 mV and – 29 mV, indicating sufficient stabilization to assure long-term stability. Even though the system is mainly sterically stabilized (Poloxamer 188 is a non-ionic surfactant), there was apparently also some contribution of electrostatical stabilization, since absolute value of zeta potential was quite high. High absolute value of zeta potential with this stabilizer has been reported previously in a study where PLGA nanoparticles were covered by Poloxamer 188 and zeta potential decreased from -10 to -20 mV after covering the particles (43). Another

more probable reason for negatively charged glibenclamide nanocrystals is the fact that glibenclamide is a weakly acidic drug, with pKa value around 5.1, therefore, it ionize at neutral pH (35, 44).

Further characterisation was implemented using nanocrystal samples NC_{10}, NC_{40} and NC_{60}. Sample NC_{10} with the smallest amount of stabilizer was chosen, because it seemed this amount of stabilizer was still sufficient for adequate stabilization, even though average particle size was a bit bigger than in sample NC_{40}. Although 0.6 g of Poloxamer 188 per gram of drug (sample NC_{60}) seemed too high and particles were bigger compared to NC_{40}, we decided to perform assays also with this sample. The decision was based on the findings of redispersibility of freeze-dried samples, which was inadequate with smaller amounts of stabilizer, as explained below.

Nanocrystal samples NC_{10} and NC_{40} were freeze-dried and the success of freeze-drying process was checked by redispersibility test. If drying is successful, the dried nanoparticles should be easily redispersed in medium and no aggregation should be noticed. Freeze-dried samples were dispersed in saturated glibenclamide solution of stabilizer (prepared as described in section 3.3.1). As they did not redisperse well and nanosized particles were not achieved, PCS measurement was impossible. Implementation of a force by mixing on vortex and sonication was also not successful. Therefore, two new batches were produced: one with lactose as cryoprotectant and another one with higher amount of stabilizer (sample NC_{60}). Both options can be a solution for enhanced redispersibility of the samples according to literature data (1, 16), but unfortunately in our study did not prove to be prosperous. Nanometer size of our drug delivery system was not preserved in freeze-drying process; hence further studies are needed to develop suitable drying method for glibenclamide nanocrystals.

4.1.3. Solubility test

Stabilizer used in our study is also a solubilizing agent (39), so it may have a big impact on drug solubility. To check the influence of stabilizer on glibenclamide solubility saturated solutions of glibenclamide with different amounts of Poloxamer 188 were analysed with HPLC. The saturated concentrations obtained in this assay are shown in Table V.

Table V: Glibenclamide solubility in aqueous solutions of stabilizer (Poloxamer 188) at room temperature.

$C_{stab.}$ (% (w/v))	$C_{glib.}$ (µg/ml)
0	0.32
2	1.19
8	2.94
12	4.06

The aim of this experiment was not to compare the absolute numbers, but to see to what extent an increased amount of stabilizer improves drug solubility. It is well evident that the stabilizer has a big influence on solubility of glibenclamide and that higher amounts affect the solubility much more. Therefore, it can be expected that solubility and consequently also dissolution rate of produced nanocrystals will be improved as well, if formulation contains higher amounts of stabilizer.

4.2. Particle shape and morphology

SEM imaging was performed to determine morphology and size of glibenclamide nanocrystals. The results were compared to PCS measurements.

The additional information about the morphology was especially important in case of freeze-dried samples, since it was not possible to redisperse them to nanosized particles, even after mixing on vortex and sonication. Consequently PCS analysis was not possible, as it can measure particle size only in completely dispersed nanosized samples (2). On top of that, when the sample is heterogeneous (high PI values), the determination of particle size by PCS is not reliable anymore, as larger particles can overshadow smaller ones. Therefore, SEM imaging was carried out to check particle's morphology and to verify preservation of nanocrystal structure after freeze-drying.

Right image in Figure 9 evidently shows that nanocrystals after drying are aggregated in bigger particles. The image shows sample NC_{10}, but aggregates were formed in all freeze-dried samples. Nevertheless, when scanned closer it was clear that nanocrystals were still present as building blocks of packed structure (Figure 10).

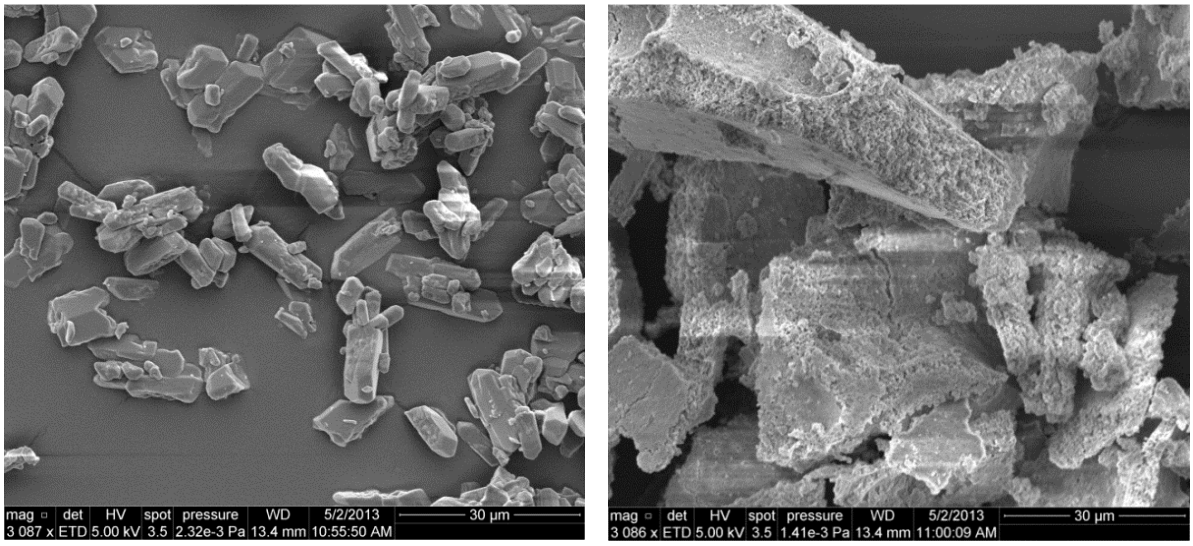

Figure 9: SEM images of glibenclamide (left) and freeze-dried nanocrystals NC_{10} (right). The scale bar represents 30 µm.

SEM image of pure glibenclamide (left image in Figure 9) was recorded in order to estimate particle size of bulk material and in this way to evaluate the effectiveness of particle size reduction in the milling process. Initial particle size of the drug was approximately from 5 to 30 µm.

Based only on images presented in Figure 9, it could be concluded that milling was not successful, since the particle size was not reduced at all, because at first sight milled particles look much bigger than the drug itself. However, close scanning revealed that crystals are uniform and nanosized in the dried sample despite the aggregates were formed (Figure 10).

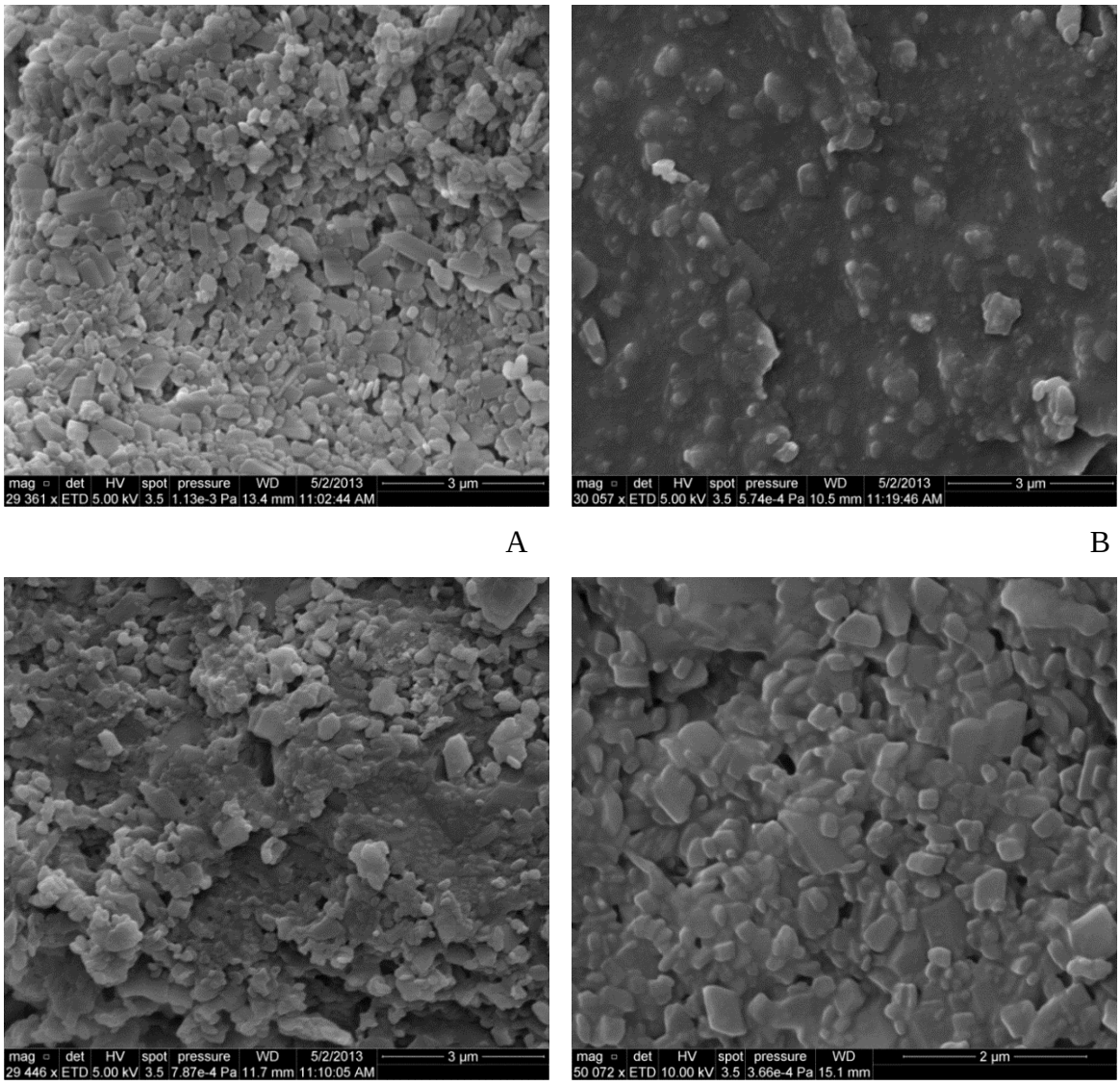

Figure 10: SEM images of nanocrystals: (A) freeze-dried NC_{10}, (B) freeze-dried NC_{60}, (C) freeze-dried NC_{40} with lactose, (D) nanosuspension of NC_{40}. The scale bars on A, B and C represent 3µm and 2µm in D.

Comparison between nanocrystal samples NC_{10} (Figure 10A) and NC_{60} (Figure 10B) revealed quite a big difference. In case of milling with 0.6 g of Poloxamer 188 per gram of drug, there is an excess of stabilizer, which is distributed freely around the particles. Consequently SEM image is a bit blurred and nanocrystals are not so clearly seen as in the image of sample containing 0.1 g of stabilizer per gram drug. Addition of lactose as a cryoprotectant in freeze-drying process has the same impact on the appearance of the particles (Figure 10C). Lactose molecules are distributed around our particles and when dispersion medium is added, lactose between particles dissolves and particles get separated, therefore, redispersibility is improved.

Figure 10D represents an image of nanocrystal sample NC_{40} in suspension, which was air dried prior SEM imaging. Particle size can be estimated to vary from approximately 150 to 500 nm. Since the sample was prepared from nanosuspension, the same as used in PCS measurement, the comparison of particle size obtained by both analyses (SEM and PCS) can be made. Average particle size of nanocrystals determined by PCS was between 260 and 300 nm and PI was around 0.2 (Table IV in section 4.1.2). The size estimated by SEM was comparable to or a bit bigger than in PCS measurements, whereas the same cannot be said for the distribution of particle size. PI values around 0.2 are indicating quite homogeneous distribution of particle size (17), but on SEM image a significant difference in particle size can be observed. Particle shape was not spherical, what can be the reason for inaccuracy in estimation of particle size and polydispersibility with PCS.

To sum up, PCS and SEM results are complementary to each other, since one reveals particle size in dispersion and the other in dry sample. The results showed increase in particle size due to aggregation of nanocrystals after drying, although nanosized particles can still be observed within these aggregates.

4.3. Physical and chemical characterisation of glibenclamide nanocrystals

4.3.1. DSC and XRPD analysis

DSC and XRPD measurements were used to characterise solid state of samples and to prove the drug in produced nanocrystals is still in crystalline form and it had not become amorphous during the milling process.

The results of DSC analysis are represented in Figure 11. Pure glibenclamide (Figure 11a) exhibits an endothermic peak at 175.31°C due to melting. According to the literature data glibenclamide has a melting point of 173-175°C (35). Stabilizer used in our study, Poloxamer 188 (Figure 11b), exhibits an endothermic peak at 54.07°C, which corresponds to its melting point being between 52 and 57°C as reported in the literature (39). Both peaks remained at almost the same temperature when analysing other samples: freeze-dried NC_{10} and NC_{40} (Figure 11e and 11f) and corresponding physical mixtures of stabilizer and drug (Figure 11c and 11d). Small shift to lower temperatures was observed, what can typically happen in the presence of another substance (22). In our case a drug and a stabilizer were present in the

sample. DSC curves of nanocrystals exhibit even larger shift compared to physical mixtures, what can additionally be explained by the influence of smaller particle size. Broadening of melting peaks is characteristic for small particles as well (23).

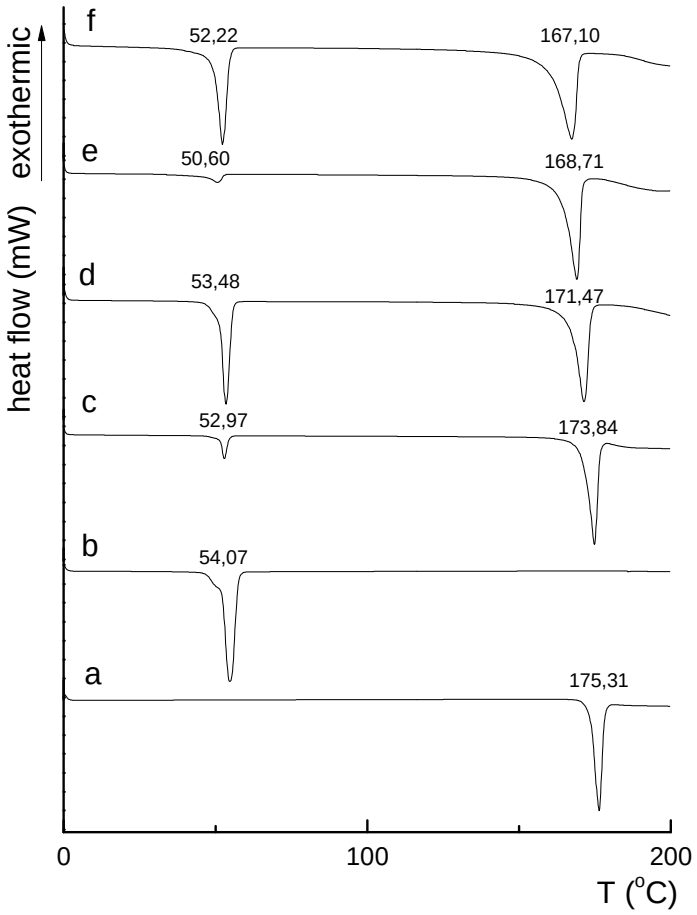

Figure 11: DSC curves of: (a) pure glibenclamide, (b) pure Poloxamer 188, (c) physical mixture corresponding to the composition of NC_{10}, (d) physical mixture corresponding to the composition of NC_{40}, (e) freeze-dried NC_{10}, (f) freeze-dried NC_{40}.

To support DSC results an XRPD analysis was carried out with nanocrystal samples NC_{40} and NC_{60}. According to XRPD results (Figure 12) it cannot be clearly stated that prepared nanocrystals (Figure 12f and 12g) were completely crystalline. There are some crystalline peaks, characteristic for glibenclamide present, but generally we cannot conclude how much it is in crystalline and how much in amorphous form. When analysing an amorphous substance a baseline shift is commonly present (Figure 12b), but this was not seen in any case of our nanocrystalline samples. Crystalline substances typically have quite flat base line with sharp peaks characteristic for the substance itself (Figure 12a).

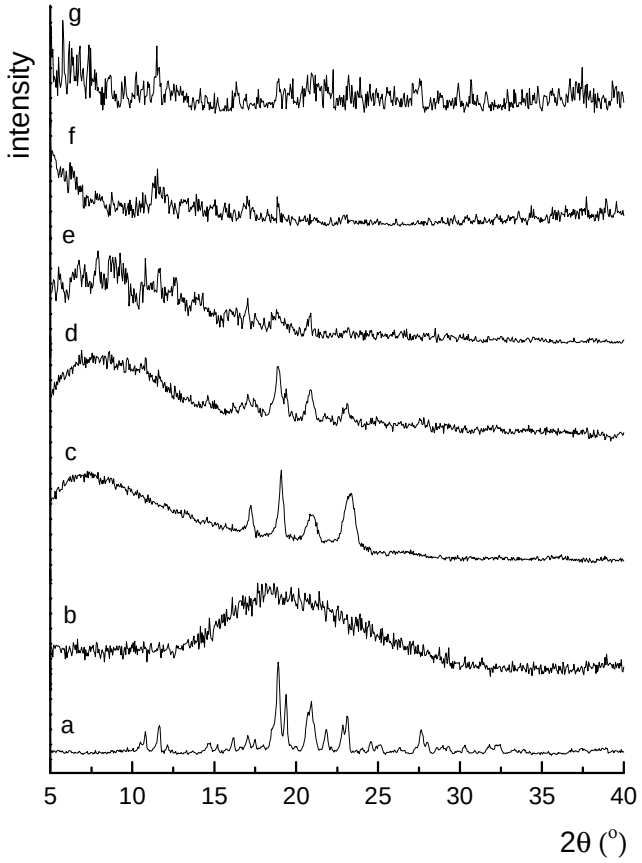

Figure 12: XRPD patterns of (a) crystalline glibenclamide, (b) amorphous glibenclamide, (c) pure Poloxamer 188, (d) physical mixture corresponding to the composition of NC_{40}, (e) physical mixture corresponding to the composition of NC_{60}, (f) freeze-dried NC_{40}, (g) freeze-dried NC_{60}.

Results show that Poloxamer 188 (Figure 12c) is partly amorphous, since the base line is uneven at the beginning, and partly crystalline because of sharp peaks in the middle. Stabilizer (Figure 12c) and glibenclamide (Figure 12a) peaks are overlapping, which complicate interpretation of results obtained with nanocrystalline samples. In the DSC curve of pure Poloxamer 188 (Figure 11b) there is only endothermic melting peak present and no other thermal event indicating presence of an amorphous form (e.g. T_g). Even though based only on DSC analysis we could conclude it is crystalline, some small parts can still be amorphous, but they are too small to be observed in DSC curve. On results of both analyses it can be concluded our stabilizer is mainly crystalline with some amorphous parts present.

Nanocrystals in XRPD graph (Figure 12f and 12g) have flat base line with many peaks, some of them being typical for glibenclamide. Therefore, we can conclude the drug is in crystalline form, even though the XRPD pattern is not completely the same as for the pure crystalline drug (Figure 12a). On top of that, it was proven that amorphous form had not been produced, since the XRPD spectrum of nanocrystals is more similar to the crystalline drug than to the amorphous (Figure 12b). Physical mixtures of stabilizer and drug (Figure 12d and 12e), which were prepared just by mixing and not by milling, behaved quite the same as our nanocrystals, prepared by milling. Therefore, we can conclude no transformation to amorphous form have been made during the milling process. We need to bear in mind also the fact that characteristic XRPD peaks usually become lower and wider when analysing material with very small particles. The spectrum is more similar to amorphous substance when particle size is below 1 μm (24). This holds true also for our case, since our particles were nanosized. Crystallinity of drug was also confirmed by DSC results (Figure 11), where only melting peak typical for crystalline form of glibenclamide, without any other thermal events common for amorphous material, was observed. It was proven with both methods that our samples are crystalline, indicating amorphous form, which quicker leads to stability related problems, has not been generated during milling process.

4.3.2. Raman spectroscopy

Raman spectroscopy was performed during intrinsic dissolution rate analysis with flow-through cell setup in order to detect any changes in crystallinity, while the formulation was exposed to the medium. Transformation from crystalline to amorphous form was not very probable and expected, but possible changes in polymorphic form during dissolution could have occurred. A Raman spectrum of pure drug was recorded successfully (Figure 13a), while nanocrystals showed fluorescence due to the presence of fluorescent stabilizer (Figure 13b). Fluorescence has much higher intensity (25), thus broad fluorescence bend appeared overwhelming Raman signal of investigated nanocrystal formulation. The spectrums obtained were therefore not useful for interpretation.

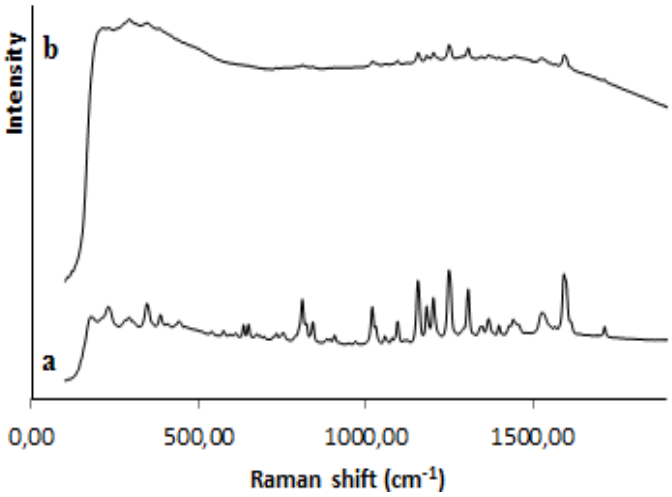

Figure 13: Raman spectrums of (a) pure glibenclamide and (b) nanocrystals NC_{40} obtained during intrinsic dissolution rate analysis with flow-through cell setup.

4.3.3. Non-linear optical imaging

This analysis was performed in order to visualize the solid state of our samples and to see how drug and stabilizer are distributed within the structure of nanocrystals. At the same time two images were recorded, one represents CARS signal and the other SHG signal. CARS signal gives information about the presence of the drug and based on SHG signal it can be determined whether the sample is crystalline or amorphous, as explained below.

During imaging the sample was firstly irradiated by 2 lasers to obtain images (CARS and SHG). Then each of the lasers was switched off separately, so images were recorded with one laser only. CARS and SHG signals can be seen when both lasers are on, but when one laser is switched off, the CARS signal should not be seen. If it is still possible to detect CARS signal, then fluorescence is present. Due to its influence on the signal, no conclusion can be made. If the SHG signal is present with one laser, then sample is crystalline, if not, it is amorphous, but only if there is no fluorescence.

It is possible to distinguish between different compounds present in the sample, if they give a signal at different wavelengths. However, Poloxamer 188 gives the response at the wavelength characteristic for glibenclamide, thus it was not possible to differ between the drug and stabilizer. If we wanted to perform further analysis with this technique, scanning of whole spectrum of the drug and the stabilizer separately was necessary. Based on these results, two other wavelengths specific only for glibenclamide can be chosen. However, this means a lot of additional work and because this analysis is not of crucial importance for our

characterisation, further imaging was not performed. As already mentioned, with this technique it is possible to observe the solid state characteristics, but only if the sample is not fluorescent. All our samples showed fluorescence due to the presence of fluorescent stabilizer, therefore no conclusions regarding crystallinity of the samples could have been made. An example of glibenclamide nanocrystals image is presented in Figure 14.

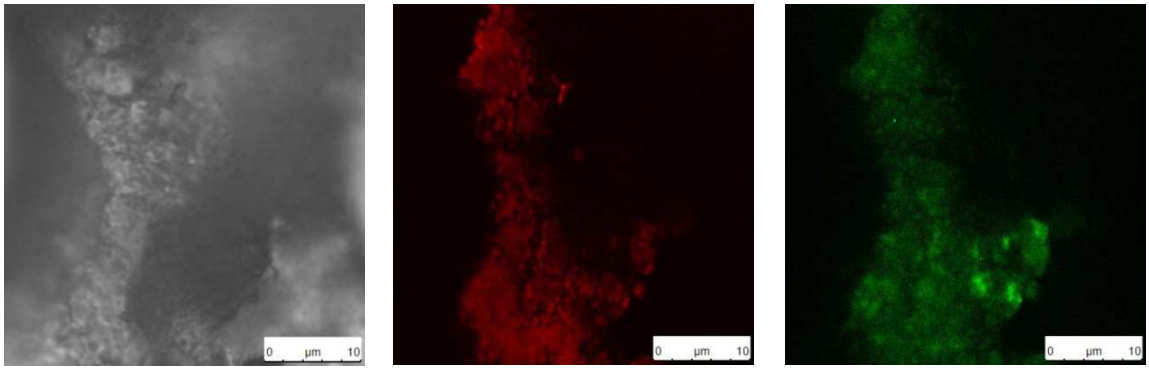

Figure 14: Bright field image (left), CARS image (middle) and SHG image (right) of glibenclamide nanocrystals NC_{60}. The scale bar represents 10 µm.

4.3.4. Evaluation of chemical stability

Due to high energy input during milling process not only polymorphic changes may occur, but also chemical degradation of the drug. To make sure no chemical changes occurred in glibenclamide structure, HPLC analysis of nanocrystals was performed. Typical chromatogram is presented in Figure 15.

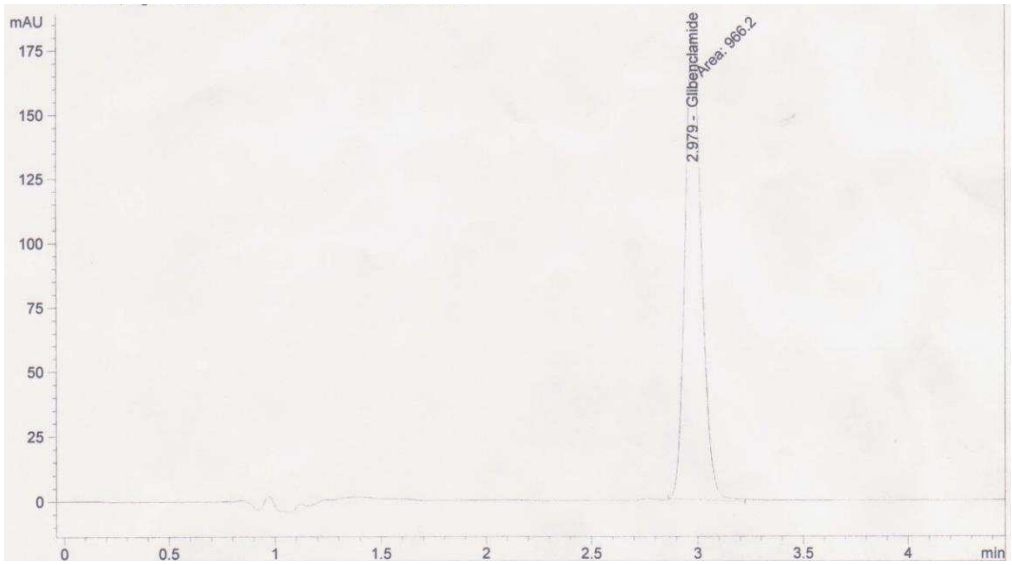

Figure 15: Chromatogram of nanocrystal sample NC_{40}. Abscissa represents time (min) and ordinate intensity (arbitrary units).

Since no additional peaks were observed, which would indicate the presence of other compounds as a result of degradation, chemical stability of glibenclamide during the milling process was confirmed.

4.4. Dissolution test

Dissolution test was performed with nanocrystals in suspension and with freeze-dried samples with or without cryoprotectant. In each group nanocrystals containing 0.1, 0.4 and 0.6 g of stabilizer per gram of glibenclamide were tested. Dissolution profiles of all nanocrystal samples, physical mixture containing 0.6 g of stabilizer and 1 g of drug and pure glibenclamide are presented in Figures 16, 17 and 18.

Nanocrystals in suspension (Figure 16) dissolved completely very quickly, as the concentration plateau was reached within the first sampling interval (30 s) already. The dissolution of physical mixture of stabilizer and drug was slower, around 90% of drug dissolved in 1 h. The dissolution of pure glibenclamide was the slowest, not even half of the drug dissolved during the assay. The total amount dissolved from physical mixture was bigger than in case of pure drug, indicating the influence of stabilizer on dissolution of the drug due to its solubilizing effect.

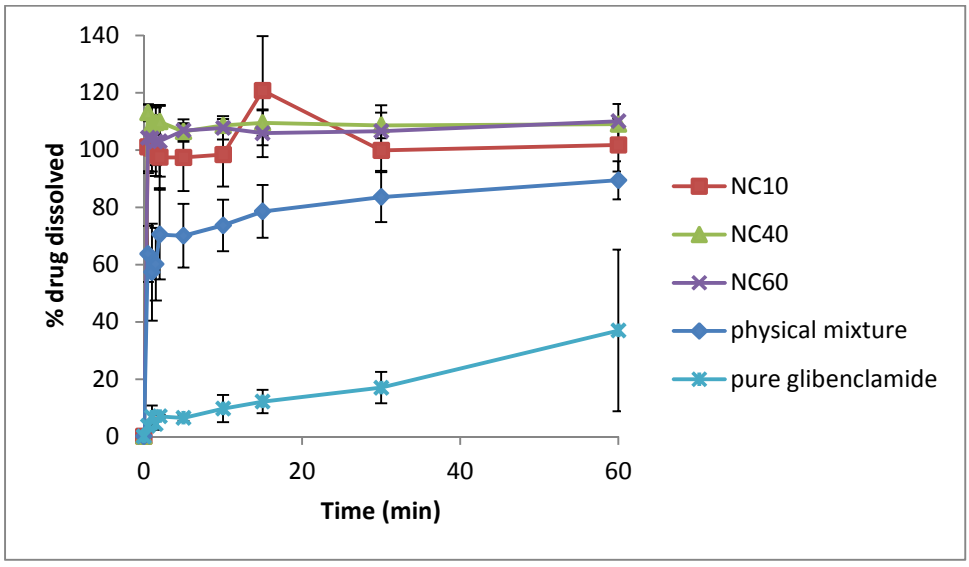

Figure 16: Dissolution profiles of nanosuspensions NC_{10}, NC_{40} and NC_{60}, physical mixture of stabilizer and drug corresponding to the composition of NC_{60} and pure glibenclamide. The amount of drug dissolved is expressed as a fraction of theoretical amount (5mg) used in dissolution test.

The total amount of drug dissolved in nanosuspensions was above 100%, since the calculations were based on theoretical content of the drug in nanocrystal samples (5 mg), but the drug concentration was probably higher due to water evaporation during the milling process. We have chosen 5 mg dose of glibenclamide for our studies, since this is a therapeutic dose used for the treatment of diabetes mellitus type II (45). The amount of the drug used assured the sink conditions, being important, because we did not want that drug already dissolved influenced on dissolution of undissolved material.

All nanocrystals exhibited faster dissolution compared to pure drug and also physical mixture of stabilizer and drug, so we can come to a conclusion that our research has been successful. According to the results obtained, the primary aim of our study, improving the dissolution rate of glibenclamide, has been achieved, at least with samples in suspension form.

The dissolution profiles of freeze-dried nanocrystals, pure drug and physical mixture of stabilizer and drug are presented in Figure 17.

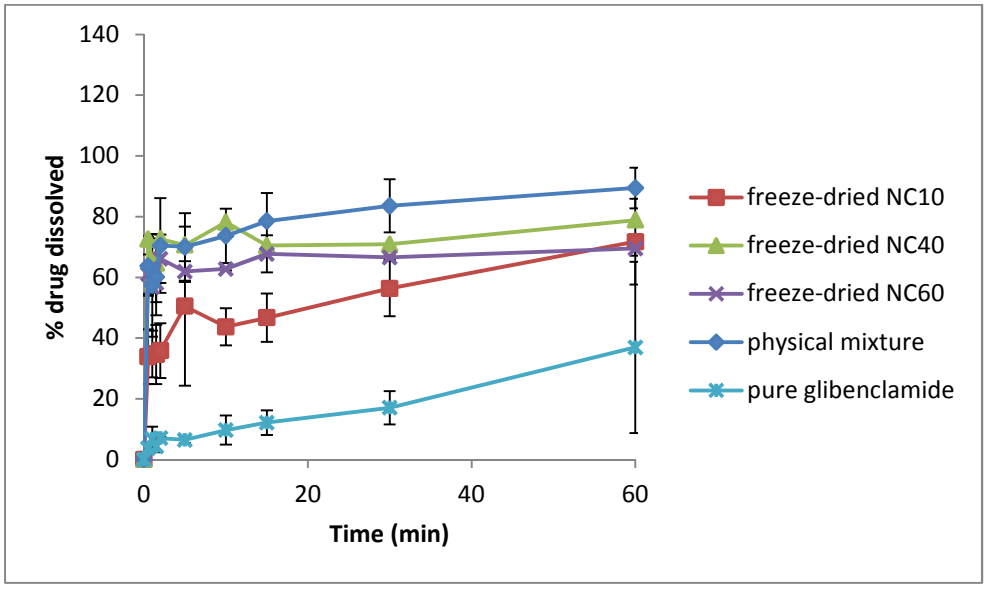

Figure 17: Dissolution profiles of freeze-dried nanocrystals NC_{10}, NC_{40} and NC_{60}, physical mixture of stabilizer and drug corresponding to the composition of NC_{60} and pure glibenclamide. The amount of drug dissolved is expressed as a fraction of total drug amount determined in HPLC analysis.

All dissolution profiles are lower than the one of physical mixture of stabilizer and drug, which indicates that the improvement in dissolution of glibenclamide was lost due to the drying process. As already described, dried nanocrystals did not redisperse to nanosized particles when medium was added, most probably due to aggregation during water removal. The structure of freeze-dried material changed and nanocrystallinity was lost in drying process. Therefore, the dissolution of freeze-dried samples was slower and less drug dissolved during the assay compared to samples in suspension form (Figure 16). Nevertheless, higher amounts of glibenclamide dissolved in all dried samples compared to pure drug, probably due to the presence of stabilizer with solubilizing properties.

Freeze-dried nanocrystals NC_{40} and NC_{60} have quite comparable dissolution profile, whereas freeze-dried NC_{10} was dissolving slower. The total drug amount in sample NC_{10} did not dissolve even in 1 h. The explanation for so slow dissolution can be related to formation of very firm aggregates after drying and poor redispersibility afterwards. The amount of stabilizer used was probably not enough for proper stabilization of all nanocrystals. Better dissolution profiles of samples with higher stabilizer concentrations (NC_{40} and NC_{60}) can be related to improved solubility due to solubilizing effect of stabilizer.

When lactose was added as a cryoprotectant in freeze-drying process, the effect on dissolution profile was observed. The dissolution profiles are presented in the Figure 18. Even though the redispersibility of these samples was not sufficient to obtain sample suitable for particle size measurement with PCS, their dissolution improved. In case of freeze-dried samples NC_{40} and NC_{60} with addition of lactose complete dissolution was achieved quickly after beginning of the test. Higher amounts of glibenclamide dissolved during the assay compared to pure drug and physical mixture of stabilizer and drug. In comparison with freeze-dried samples without lactose (Figure 17) the dissolution profiles were higher, more drug dissolved, hence it can be claimed that the addition of cryoprotectant proved to be useful. Freeze-dried NC_{10} with addition of lactose was dissolving slowly within the first 30 min, similar to the dissolution of freeze-dried samples without lactose as cryoprotectant (Figure 17). Therefore, it can be claimed that the use of cryoprotectant was not proven to be so effective in this sample.

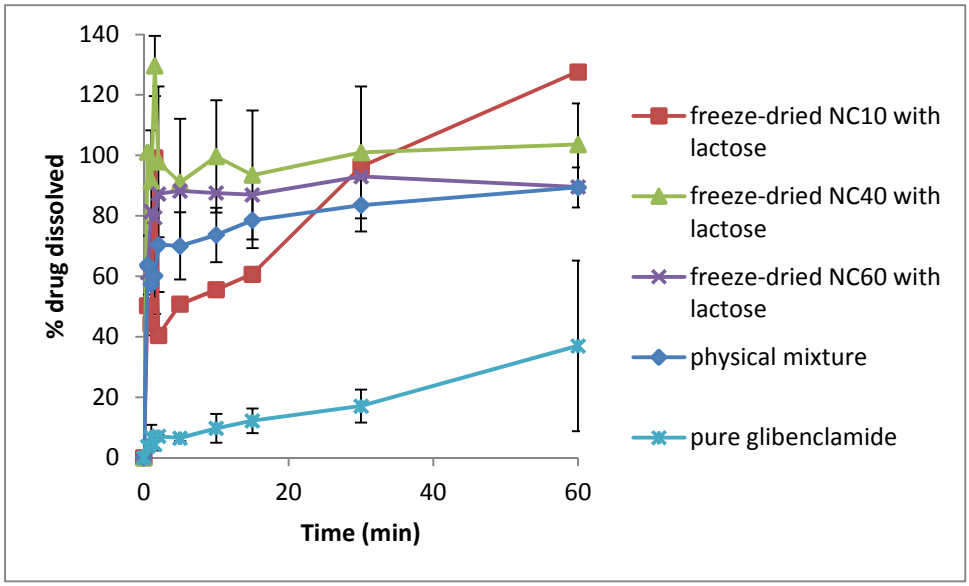

Figure 18: Dissolution profiles of freeze-dried nanocrystals $NC_{10,}$ NC_{40} and NC_{60} with lactose as a cryoprotectant, physical mixture of stabilizer and drug corresponding to the composition of NC_{60} and pure glibenclamide. The amount of the drug dissolved is expressed as a fraction of total drug amount determined in HPLC analysis.

To sum up, drug dissolution improved with all nanocrystal formulations in suspension form. However, after freeze-drying process the redispersibility was poor and consequently the dissolution rate was not enhanced either. To overcome this problem, lactose was added as a cryoprotectant. In samples with higher amounts of stabilizer (NC_{40} and NC_{60}) drug dissolution improved, but the addition of cryoprotectant was not so effective when smaller amounts of stabilizer (NC_{10}) were used.

4.5. Evaluation of intrinsic dissolution rate

The intrinsic dissolution rate of the drug nanocrystals can be correlated to their solubility (29, 30). Even though the solubility is a characteristic of the substance itself, usually it is improved with nanosized particle formation. The intrinsic dissolution rate was tested by flow-through cell method, in which the dissolution occurs from a flat and constant surface. Results of this analysis are presented graphically in Figure 19.

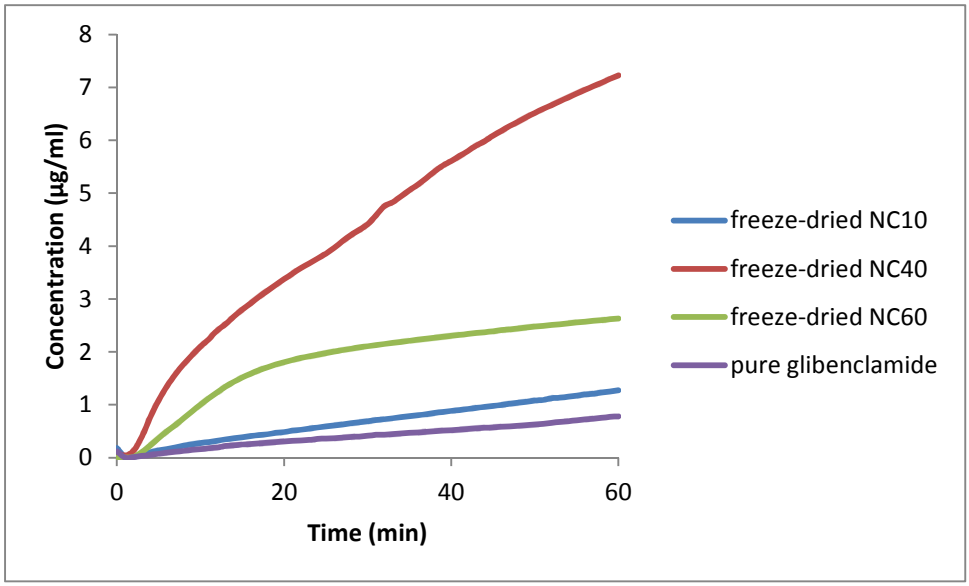

Figure 19: Dissolution profiles of freeze-dried glibenclamide nanocrystals NC_{10}, NC_{40}, NC_{60} and pure glibenclamide determined by flow-through cell analysis.

The slope of each curve needs to be considered in interpretation of the results and in comparison of different samples. The intrinsic dissolution rate of a drug in sample NC_{10} did not improve much compared to pure glibenclamide. The improvement was noticed in sample NC_{60} and even more in case of sample NC_{40}. The observation is in line with previous findings, where 0.4 g of stabilizer per gram of glibenclamide (NC_{40}) represented an optimal amount to achieve the smallest particles with a little bit better dissolution profile compared to samples with smaller (NC_{10}) or higher amounts (NC_{60}) of stabilizer.

Physical mixtures of stabilizer and drug have also been tested, but unfortunately the results were not repeatable and useful. A reason can be ineffective mixing due to different drug's and stabilizer's particle size resulting in inhomogeneous sample. Therefore, the results are presented in comparison to pure glibenclamide and not to physical mixture of stabilizer and drug as usual, even though this makes the evaluation less reliable. Since the stabilizer has also an effect on drug solubility and the results are not expressed in comparison to physical mixture of stabilizer and drug, the solubilities of glibenclamide in aqueous solutions with different stabilizer concentrations (Table V in section 4.1.3) need to be taken into account when interpreting result of flow-through cell assay.

The solubility test (section 4.1.3) revealed that our stabilizer i.e. Poloxamer 188 has a big influence on solubility of the drug. When higher amounts were used, the effect was much more prominent. Nevertheless, the concentrations in flow-through cell test were much lower

than in the separate solubility study, since the buffer was continuously circulating through the system and diluting the drug and stabilizer already dissolved. Because of lower concentrations of glibenclamide present during this assay, the influence of the stabilizer on drug solubility was less expressed than in separate solubility test, but it was still present. Based on our results, we cannot conclude to what extent improved solubility was due to solubilizing characteristics of Poloxamer 188 and how much due to nanometer size of particles achieved by milling.

However, it can be concluded that the improvement in intrinsic dissolution rate, which can be correlated to solubility, was not solely due to solubilizing effect of stabilizer but also due to formation of nanosized particles. The solubility of glibenclamide, determined in separate solubility test (section 4.1.3), was greater in aqueous solutions with higher stabilizer concentrations. Hence, if only the amount of stabilizer influenced on drug solubility, the dissolution profile of nanocrystals NC_{60} would be higher than the profile of nanocrystals NC_{40} due to higher amount of stabilizer present in the sample. But the opposite occurred i.e. smaller nanocrystals in sample NC_{40} exhibited higher solubility in flow-through cell measurement. Therefore, it can be concluded that not only solubilizing properties of Poloxamer 188, but also the presence of nanosized particles influenced on improved solubility.

Another reason for improved solubility could be formation of amorphous material, which could have been generated during the milling process. However, in our study it has been proved by DSC and XRPD analyses that our particles were crystalline or the amount of amorphous material is below detection limit. Therefore, it can be claimed that improved solubility was mainly the consequence of nanosized particles.

4.6. Evaluation of physical stability of nanocrystal dispersions

Nanocrystalline samples NC_{10}, NC_{40} and NC_{60} in suspension were regularly analysed using PSC during 2 months period. Measurements of average particle size, polydispersity index and zeta potential were performed straight after production and after 1, 2, 4 and 8 weeks.

Results of particle size measurements are presented in Figure 20. Average particle size remained almost the same after 2 months storage in all the samples, also in sample containing the smallest amount of stabilizer (NC_{10}). However, particle size standard deviation of this

sample increased with time, what indicates the sample became more heterogeneous, which could result in long-term instability. In sample NC_{40} smaller particles were present (260 – 270 nm) compared to sample NC_{60} (around 280 nm), even though smaller amount of stabilizer had been used. Probably the amount of Poloxamer 188 used in NC_{60} was too high and the reverse effect occurred – particles were larger contrary to our expectation due to bigger amount of stabilizing agent used. Another reason for this could also be thicker layer of stabilizer around same-sized drug core, what made particle a bit bigger. More stabilizer around the particle attracts more water and consequently its hydrodynamic size measured by PCS is bigger.

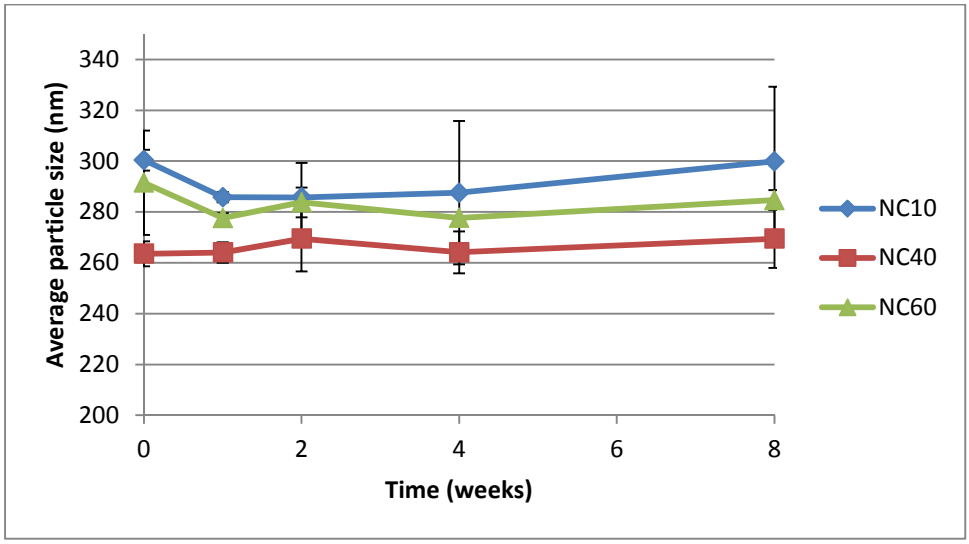

Figure 20: Average particle size of glibenclamide nanocrystals NC_{10}, NC_{40} and NC_{60} over time period of 8 weeks.

Beside average particle size PI was also measured for all the samples in the same time points. The results are presented in Figure 21. For samples NC_{40} and NC_{60} PI values were around 0.2 after 2 months. The value is comparable to PI right after preparation, so it can be concluded that particles did not aggregate during storage and their particle size was still as homogeneous as straight after production. However, the same cannot be claimed for nanocrystals NC_{10}, as their PI increased. It was above 0.25 after 4 weeks already and even higher after 8 weeks. This is the evidence that 0.1 g of stabilizer per gram of glibenclamide was not enough for a proper stabilization of produced nanocrystals. If standard deviations are also considered, no conclusions about any difference between all the samples can be made, except that PI did not evidently change in 2 months.

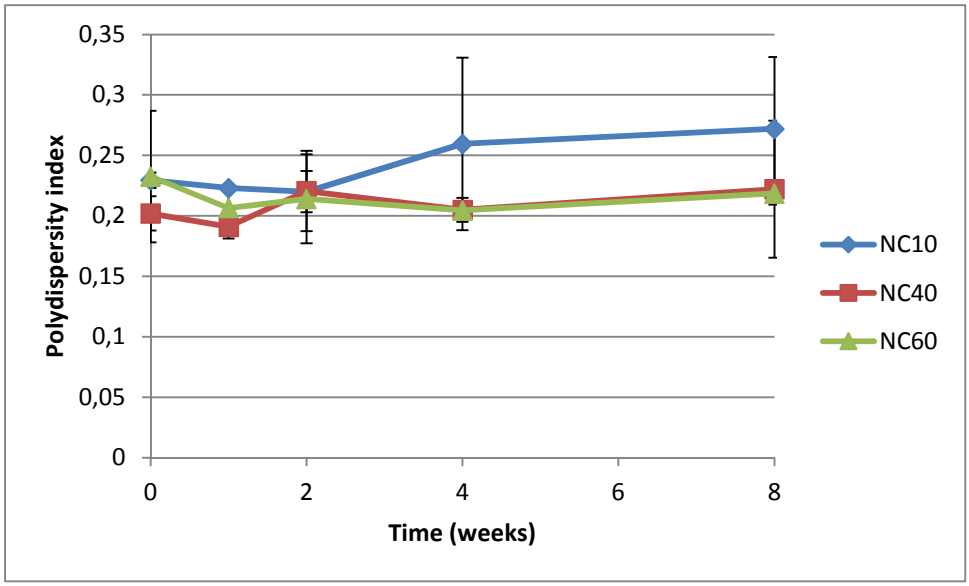

Figure 21: Polydispersity index (PI) of glibenclamide nanocrystals NC_{10}, NC_{40} and NC_{60} over time period of 8 weeks.

Zeta potentials of nanocrystals are presented in Figure 22. All the samples showed zeta potential between – 20 and – 30 mV straight after milling and also after 2 months period. Zeta potential is negative mainly because of the ionization of acid functional groups of glibenclamide at neutral pH (44). When stabilization is based on formation of sterical barriers among particles as in our study, zeta potential is not an appropriate parameter for the estimation of long-term stability (1). Due to relatively high absolute values of zeta potential and additional presence of steric stabilizer on nanocrystal surface, we can conclude our formulation was physicaly stable in time of 8 weeks.

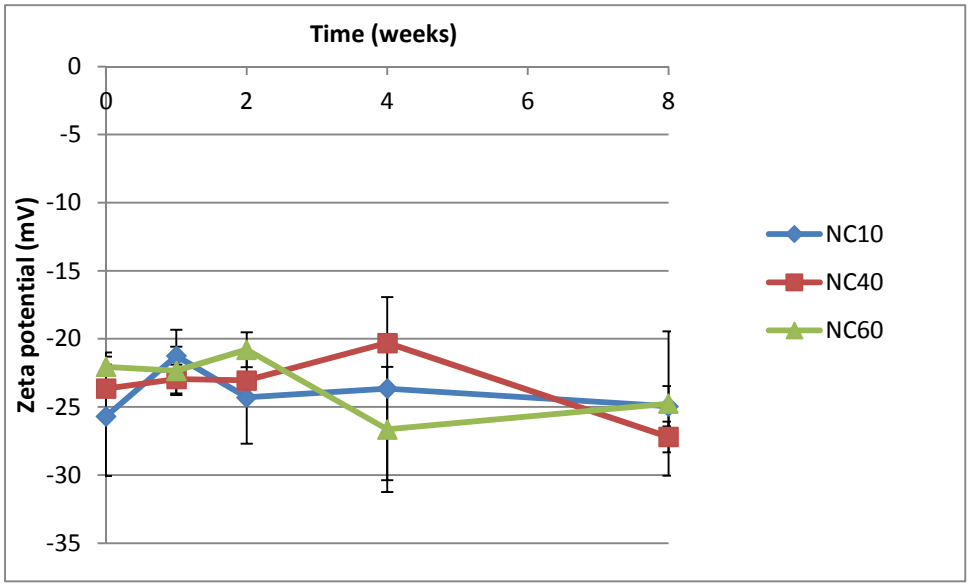

Figure 22: Zeta potential of glibenclamide nanocrystals NC_{10}, NC_{40} and NC_{60} over time period of 8 weeks.

Chemical stability was also investigated to see if there had been some changes in glibenclamide structure during storage. Based on chromatogram obtained with HPLC measurement no chemical degradation of the drug was noticed, since no additional signals were observed.

In general we can conclude that produced nanocrystals were physically and chemically stable in suspension for at least 2 months. Only nanocrystals containing the smallest amount of stabilizer (NC_{10}) showed small increase in PI at the end of this stability study.

5. CONCLUSION

Glibenclamide nanocrystals were successfully produced with pearl milling technique using different amounts of Poloxamer 188 as a stabilizing agent. Average particle size was below 300 nm with PI around 0.2, indicating quite homogeneous size distribution. Our nanodelivery system was mainly sterically stabilized, however zeta potential of nanocrystals (between -20 and -30 mV) also contributed to their long-term stability.

In freeze-drying process aggregates were formed, confirmed by SEM. Consequently, dried nanocrystals did not redisperse to nanosized particles. DSC and XRPD results showed that glibenclamide remained in crystalline form and no chemical degradation was noticed after milling process, as confirmed by HPLC. Raman spectroscopy and non-linear optical imaging were not useful to determine crystallinity due to the presence of fluorescent stabilizer.

The improvement in dissolution rate of glibenclamide was achieved with nanosizing, especially with nanocrystals in suspension. Dried nanocrystals were dissolving slowly, but lactose used as cryoprotectant has proved to be efficient in enhancing drug dissolution, even though the redispersibility was not improved enough to enable determination of particle size by PCS analysis. Intrinsic dissolution rate of glibenclamide nanocrystals, which can be correlated to their solubility, improved when nanosized particles were formed, as confirmed by flow-through cell method. Also the presence of Poloxamer 188 with its solubilizing properties contributed to improved solubility of prepared nanocrystals.

Physical stability, evaluated by average particle size, PI and zeta potential measurements, revealed that produced glibenclamide nanocrystals were stable in suspension for at least 2 months, especially when higher percentages of stabilizer were used.

In general, we can claim our research was successful, as the improvement in solubility and dissolution rate of glibenclamide has been achieved by formation of nanocrystals with pearl milling technique.

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