UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

Valentina Božič

MAGISTRSKO DELO

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJE

Ljubljana, 2016

UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

Valentina Božič

IZDELAVA IN VREDNOTENJE NANOSUSPENZIJ DEKSAMETAZONA ZA DERMALNO UPORABO

PREPARATION AND CHARACTERIZATION OF DEXAMETHASONE NANOSUSPENSIONS FOR DERMAL APPLICATION

Ljubljana, 2016

Master thesis was written based on a research work done from April 2014 to August 2014 at Institute of pharmaceutical technology, Free University Berlin, Germany, under co-mentorship of Prof. Dr. Roland Bodmeier and mentorship Assist. Prof. Dr. Petra Kocbek.

ACKNOWLEDGEMENT

I would like to give my special appreciation to my co-mentor Prof. Dr. Roland Bodmeier, who kindly accepted me in his research group and to my working mentors Ph.D. students Benjamin Balzus and Luisa Höhne for their support during my research time in the laboratory.

I would also like to thank to the research team at the institute from whose extensive knowledge and friendship I have benefited more than words can say.

I would particularly like to express my gratitude to my mentor Assist. Prof. Dr. Petra Kocbek, who patiently helped me bring this thesis to a successful close.

Finally, I must thank my family for support during the whole study, my friends for making these years unforgettable and one special person for encouragement and inspiration.

STATEMENT

I hereby declare that this master thesis was done by me under supervision of Assist. Prof. Dr. Petra Kocbek and co-supervision of Prof. Dr. Roland Bodmeier.

Valentina Božič

COMMITTEE

Head of Committee: Assoc. Prof. Dr. Mitja Kos

Mentor: Assist Prof. Dr. Petra Kocbek

Co-mentor: Prof. Dr. Roland Bodmeier

Member of Committee: Dr. Rok Frlan

TABLE OF CONTENTS

POVZETEK

Zmanjševanje velikosti delcev učinkovine je eden od pristopov, ki se dandanes uporabljajo v farmacevtskem razvoju za izboljšanje lastnosti težko topnih učinkovin. Fizikalno-kemijske lastnosti učinkovin se spremenijo, če zmanjšamo velikost delcev. Zaradi svojih prednosti (npr. izboljšana topnost in hitrost raztapljanja, povečana adhezivnost in penetracija) se nanosuspenzije že uporabljajo v nekaterih farmacevtskih in kozmetičnih dermalnih formulacijah; kljub temu pa je večina raziskav še vedno osredotočena na njihovo peroralno in intravensko uporabo.

Namen te raziskave je bil pripraviti in ovrednotiti formulacije nanosuspenzij z deksametazonom za dermalno uporabo. Za zmanjšanje velikosti delcev smo izbrali metodo mokrega mletja v krogličnem mlinu.

Nanosuspenzije smo pripravili z uporabo dermalno sprejemljivih stabilizatorjev tj. poloksamera 188 in 407, hidroksilpropilmetilceluloze (HPMC) in vitamin E polietilenglikol sukcinata (vitamin E TPGS). Nanosuspenzije smo ovrednotili glede na velikost delcev (fotonska korelacijska spektroskopija, laserska difrakcija, svetlobna mikroskopija) in zeta potencial (laserska Dopplerjeva anemometrija). Test kratkoročne stabilnosti, s katerim smo ovrednotili fizikalno stabilnost nanosuspenzij, smo izvedli pri treh različnih temperaturah (4 °C, 25 °C, 40 °C). Stabilizatorje in njihove kombinacije smo razvrstili v štiri skupine glede na njihovo sposobnost, da ohranijo fizikalno stabilnost nanosuspenzij, ki smo jo spremljali 30 dni (od najbolj do najmanj stabilne): I. HPMC in kombinacije z vitaminom E TPGS, II. poloksamer 407, III. vitamin E TPGS in kombinacije s poloksamerom 188 in 407 in IV. poloksamer 188. Najmanjšo povprečno velikost delcev smo določili v nanosuspenziji stabilizirani s poloksamerom 407 (~300 nm, PI do 0,3), z zeta potencialom okrog -22 mV.

Vodne nanosuspenzije so podvržene mikrobiološki kontaminaciji, zato je pomembno, da za ohranjanje mikrobiološke kakovosti izberemo ustrezen konzervans, ki pa lahko vpliva tudi na fizikalno stabilnost nanosuspenzij. Testirali smo tri različne konzervanse: propilenglikol, sorbinsko kislino v kombinaciji s kalijevim sorbatom in benzilni alkohol. Dokazali smo, da med testiranimi konzervansi propilenglikol najmanj vpliva na fizikalno stabilnost nanosuspenzij. Kratkoročni test stabilnosti in mikrobiološki test na agarju sta potrdila, da nanosuspenzija stabilizirana s poloksamerjem 407, zahteva 16 % (m/m) propilenglikola, da je rast mikrobov popolnoma zavrta in je nanosuspenzija še vedno fizikalno stabilna.

KLJUČNE BESEDE: nanosuspenzija, deksametazon, dermalna uporaba, mikrobiološki test, mokro mletje v krogličnem mlinu

ABSTRACT

Nanosizing is one of the approaches to deal with poorly soluble drugs in commercial pharmaceutical development nowadays. Physico-chemical properties of the drug change with particle size reduction. Due to their advantages (e.g., improved solubility and dissolution rate, increased adhesiveness and penetration), nanosuspensions are already used in some pharmaceutical and cosmetic dermal formulations. However, the majority of studies is still focused on oral and intravenous administration of nanosuspensions.

The aim of this study was to prepare and evaluate nanosuspension formulations with dexamethasone for dermal administration. Pearl milling was chosen as a method for particle size reduction. Nanosuspensions were prepared using dermally acceptable stabilizers, namely poloxamer 188 and 407, hydroxylpropyl methylcellulose (HPMC) and vitamin E polyethylene glycol succinate (vitamin E TPGS). Nanosuspensions were characterized regarding their particle size (photon correlation spectroscopy, laser diffraction, light microscopy) and zeta potential (laser Doppler anemometry). Short-term stability study at three different temperatures (4°C, 25°C, and 40°C) was performed to evaluate nanosuspension physical stability. Stabilizers and their combinations were classified into four groups according to their ability to maintain nanosuspension physical stability, which was evaluated throughout 30 days. Stabilizers were classified in the following groups based on nanosuspension stability (from the most to the least stable sample): I. HMPC and its combination with vitamin E TPGS, II. poloxamer 407, III. vitamin E TPGS and its combination with poloxamer 188 and 407 and IV. poloxamer 188. Nanosuspensions stabilized with poloxamer 407 have shown the smallest average particle size (~300 nm, polydispersity index up to 0.3) and zeta potential around -22 mV, indicating good physical stability.

Nanosuspensions are usually water containing products and thus they require preservation, which can impair their physical stability. Among the tested preservatives (propylene glycol, sorbic acid/potassium sorbate and benzyl alcohol) propylene glycol was shown to have the smallest impact on the physical stability of nanosuspensions. Short-term stability test and microbiological test on agar plates confirmed that nanosuspension stabilized with poloxamer 407, required 16% (w/w) of propylene glycol to fully inhibit microbial growth. This concentration did not affect the physical stability of nanosuspension.

KEY WORDS: nanosuspension, dexamethasone, dermal application, microbiological assay, pearl milling

RAZŠIRJENI POVZETEK

Zmanjševanje velikosti delcev učinkovine je eden od pristopov, ki se dandanes uporabljajo v farmacevtskem razvoju za izboljšanje lastnosti težko topnih učinkovin. Fizikalno-kemijske lastnosti učinkovin se spremenijo, če zmanjšamo velikost delcev. Nanosuspenzija je koloidna disperzija delcev učinkovine nanometrske velikosti v tekočem mediju. Delci učinkovine so prekriti s plastjo stabilizatorja. Stabilnost nanosuspenzij je odvisna od vrste uporabljenega stabilizatorja, ki vpliva na končno velikost delcev. Ker ima vsaka učinkovina edinstvene lastnosti, potrebujemo specifično izbran stabilizator za njeno optimalno stabilizacijo. Zaradi svojih prednosti (npr. izboljšana topnost in hitrost raztapljanja, povečana adhezivnost in penetracija) se nanosuspenzije že uporabljajo v nekaterih farmacevtskih in kozmetičnih dermalnih formulacijah, kljub temu pa je večina raziskav še vedno osredotočena na njihovo peroralno in intravensko uporabo.

Nanosuspenzije lahko pripravimo z različnimi metodami, ki jih delimo na "top down" in "bottom up" metode. Pri "top down" metodah zmanjšamo velikost delcev z mokrim mletjem v krogličnem mlinu ali s homogeniziranjem pod visokim tlakom, pri "bottom up" metodah pa pripravimo nanometrske delce učinkovine iz raztopljene učinkovine z obarjanjem. V uporabi so tudi kombinacije obeh metod, ki naj bi učinkoviteje zmanjšale velikost delcev do nanometrskega območja. V naši raziskavi smo za pripravo nanosuspenzij izbrali mokro mletje v krogličnem mlinu, ki spada med "top down" metode. Pripravili smo nanosuspenzije deksametazona, ki je težko topna učinkovina iz skupine kortikosteroidov. Pogosto se uporablja kot protivnetna učinkovina, imunosupresiv, pri zdravljenju alergij in zmanjšanem delovanju nadledvične žleze. V dermatologiji se uporablja za lajšanje kroničnih kožnih bolezni.

Namen te raziskave je bil pripraviti in ovrednotiti formulacije nanosuspenzij z deksametazonom za dermalno uporabo. Proučevali smo vpliv štirih stabilizatorjev, primernih za dermalno uporabo, vpliv dodanih konzervansov, pogojev shranjevanja in časa na lastnosti nanosuspenzij.

Nanosuspenzije smo stabilizirali z uporabo dermalno sprejemljivih stabilizatorjev tj. poloksamera 188 in 407, hidroksilpropilmetilceluloze (HPMC) in vitamin E polietilenglikol sukcinata (vitamin E TPGS). Nanosuspenzijam smo ovrednotili velikost delcev (fotonska korelacijska spektroskopija, laserska difraktometrija, svetlobna mikroskopija) in zeta

potencial (laserska Dopplerjeva elektroforeza). Najbolj učinkovito zmanjšanje velikosti delcev smo opazili pri nanosuspenziji, ki smo jo stabilizirali s kombinacijo vitamina E TPGS in poloksamera 407. Povprečna velikost delcev v tej nanosuspenziji je bila 334 nm in PI ~0,3, kar predstavlja relativno homogeno porazdelitev velikosti delcev.

Test kratkoročne stabilnosti smo izvedli pri treh različnih temperaturah (4 °C, 25 °C, 40 °C) z namenom ovrednotenja fizikalne stabilnosti nanosuspenzij. Stabilizatorje in njihove kombinacije smo razvrstili v štiri skupine glede na njihovo sposobnost, da ohranijo fizikalno stabilnost nanosuspenzij, ki smo jo spremljali 30 dni.

V prvo skupino smo uvrstili HPMC in njeno kombinacijo z vitaminom E TPGS, ki so zagotavljali najboljšo fizikalno stabilnost izdelanih nanosuspenzij. Nanosuspenzije so izkazale kratkoročno fizikalno stabilnost kljub nižjemu absolutnemu zeta potencialu (~8 mV) v primerjavi z nanosuspenzijami, kjer smo uporabili druge stabilizatorje. Fizikalno stabilnost je zagotovljala sterična stabilizacija s HPMC, ki je vplivala tudi na viskoznost vodnega medija nanosuspenzij, kar je zmanjšalo raztapljanje in agregacijo delcev. Slaba stran uporabe HPMC kot stabilizatorja je bila večja povprečne velikosti delcev po mletju (okoli 600 nm). Za dodatno zmanjšanje povprečne velikosti delcev bi potrebovali daljši čas mletja in s tem bi porabili tudi več energije. V drugo skupino smo uvrstili poloksamer 407, ki je omogočil izdelavo nanosuspenzije z najmanjšo povprečno velikostjo delcev (~300 nm, PI do 0,3) in zadosten zeta potencial za zagotavljanje kratkoročne fizikalne stabilnosti (-22 mV). Rezultati laserske difraktometrije in svetlobne mikroskopije so po enem mesecu shranjevanja pokazali prisotnost agregatov v vzorcih z dodanim konzervansom. V tretjo skupino stabilizatorjev smo uvrstili vitamin E TPGS in njegove kombinacije s poloksamerom 188 in 407. V vseh vzorcih smo opazili rast delcev z višjo temperaturo shranjevanja (fotonska korelacijska spektrometrija, laserska difraktometrija). Zadnji proučevan stabilizator, poloksamer 188, smo uvrstili v četrto skupino, saj smo opazili v nanosuspenzijah stabiliziranih s tem stabilizatorjem znatno povečanje povprečne velikosti delcev in porazdelitve velikosti delcev v testu kratkoročne stabilnosti, še posebej pri 4 °C. Zeta potenciali so nakazovali potencialno dobro fizikalno stabilnost teh nanosuspenzij, vendar pa so meritve pokazale široko porazdelitev velikosti delcev in prisotnost agregatov.

Vodne nanosuspenzije so podvržene mikrobiološki kontaminaciji, zato je pomembno, da za ohranjanje mikrobiološke kakovosti izberemo ustrezen konzervans, ki pa lahko vpliva tudi

na fizikalno stabilnost nanosuspenzij. Proučevali smo tri različne konzervanse: propilenglikol, sorbinsko kislino v kombinaciji s kalijevim sorbatom in benzilni alkohol. Nanosuspenzija stabilizirana s poloksamerom 407 in konzervirana s propilenglikolom je bila najbolj fizikalno stabilna disperzija. Dokazali smo, da med testiranimi konzervansi propilenglikol najmanj vpliva na fizikalno stabilnost nanosuspenzij. Koncentracija do 16 % (m/m) ni imela vpliva na stabilnost nanosuspenzij. Poleg vpliva konzervansa na stabilnost nanosuspenzij lahko adsorpcija konzervansa na površino delcev v nanosuspenziji povzroči zmanjšano učinkovitost ali celo odsotnost učinka konzervansa, kar smo testirali z mikrobiološkim testom.

Z mikrobiološkim testom na agarju smo ugotovili, da moramo nanosuspenzijo stabilizirano s poloksamerjem 407 konzervirati z vsaj 16 % (m/m) propilenglikola, da popolnoma preprečimo rast bakterij v časovnem obdobju štirinajstih dni. V primeru nanosuspenzij stabiliziranih s poloksamerom 407, ki smo jih konzervirali s sorbinsko kislino/kalijevim sorbatom in benzilnim alkoholom nismo zaznali mikrobne rasti vsaj osem dni.

Spektrofotometrične meritve so pokazale, da je bilo najmanj raztopljene učinkovine prisotne v vodni fazi nanosuspenzij stabiliziranih s HPMC, več v nanosuspenzijah stabiliziranih s poloksamerom 407 in največ v nanosuspenzijah stabiliziranih s poloksamerom 188. V vzorcih nanosuspenzij, ki smo jih shranjevali pri 40 °C, je bilo več učinkovine raztopljene v vodnem mediju v primerjavi z nanosuspenzijami, ki smo jih skladiščili pri nižjih temperaturah. Nadalje je bila koncentracija raztopljene učinkovine večja v nanosuspenzijah konzerviranih s propilenglikolom v primerjavi z nanosuspenzijami brez konzervansa.

Naša raziskava je pokazala, da je med proučevanimi formulacijami nanosuspenzij najbolj optimalna nanosuspenzija z 2,0 % deksametazona stabilizirana z 0,8 % (m/m) poloksamera 407 in konzervirana s 16 % (m/m) propilenglikola. Kratkoročni test stabilnosti in mikrobiološki test na agarju sta potrdila ustrezno fizikalno in mikrobiološko stabilnost izdelane nanosuspenzije.

LIST OF USED ABBREVIATIONS

BA	Benzyl alcohol
C-	Control, negative
C+	Control, positive
HPH	High pressure homogenization
HPMC	Hydroxypropyl methyl cellulose
i.v.	Intravenous application
IUPAC	International Union of Pure and Applied Chemistry
LD	Laser diffractometry
NS	Nanosuspension
P	Preservative added
P188	Poloxamer 188
P407	Poloxamer 407
PCS	Photon correlation spectroscopy
PG	Propylene glycol
PI	Polydispersity index
PR	Probe
Rpm	Rotations per minute
SA	Sorbic acid
SEM	Scanning electron microscopy
TAMC	Total aerobic microbial count
TEM	Transmission electron microscopy
TPGS	Tocopheryl polyethylene glycol succinate
TYMC	Total combined yeasts/moulds count
USP	United States Pharmacopoeia
UV	Ultra violet
WP	Without preservative added
ZP	Zeta potential

1 INTRODUCTION

1.1 Definition of nanosuspension and nanocrystals

Nanosuspension is a submicron colloidal dispersion of pure drug particles in liquid medium without any matrix forming material. Particles are covered with a layer of stabilizer. Nanosized drug particles can be in crystal (nanocrystals) or amorphous form. According to the general definition of nanoparticles, the size of drug particles in nanosuspension is below one μm, although the mean diameter is usually 200-400 nm (1, 2, 3). Nanosuspensions can be postprocessed into various types of solid dosage forms such as tablets and capsules or incorporated in semisolid dosage forms such as ointments and creams (5).

1.2 Benefits of nanosuspensions or nanocrystals in drug delivery

Many drugs are nowadays difficult to formulate into a suitable dosage form. The most of the issues originate from their low solubility, since 60% of drugs are poorly soluble in water (5, 6). Other subsequent concerns are poor bioavailability, lack of dose-response proportionally, fed/fasted variation in bioavailability, insufficient *in vitro* stability (shelf life), too short *in vivo* stability (half-life), use of undesirable excipients and co-solvents, use of extreme basic or acidic conditions, too large injection volume for i.v. administration and lack of large scale production (5, 7). Common approaches to improve solubility of poorly soluble drugs are drug conversion into a salt form, pH adjustment, application of co-solvents, cyclodextrines, formation of micelles, emulsions etc. (2).

Formulation of an innovative nanodelivery system, namely nanosuspensions, change physico-chemical properties of poorly soluble drug and improve its delivery. There has been some research performed on nanosuspensions and nanocrystals already, however, the most of attention has been given to improved oral and intravenous drug delivery. The area of dermal nanocrystal application has been slightly left behind (8).

Several problems of dermal drug delivery of conventional formulations range from low drug uptake to unwanted systemic side effects. Control over the precise drug amount that reaches different skin layers is difficult as well. Nanosuspensions can enhance the penetration of active compounds and deliver them to the targeted site (9). Not only poorly soluble drugs can be formulated as nanosuspensions, but also “medium soluble” drugs exhibit some benefits as nanocrystals in dermal drug delivery (10).

Dissolution rate and saturation solubility of nanosized drug particles are proportional to the surface area of the drug. They both increase by decreasing the particle size. Adhesiveness is improved with smaller particle size and thus a prolonged contact time at the site of administration can be achieved (3, 11, 12). To sum up, preparation of the drug in the form of nanocrystals allows control over the pharmacokinetics of the dosage form (5). A few reports exist showing that half of the dose of drug in form of nanosized particles results in similar exposure in rats than a double dose of drug in form of micronized suspension (2, 4). Dermal application of nanosuspension containing products can enhance the penetration of the drug into the skin. The increased saturation solubility of nanosized drug particles results in an increased concentration gradient and better nanosized particle adhesiveness allows a better contact with the skin which contributes to better drug penetration (Figure 1). Moreover, with nanocrystal formulations a drug penetration through hair follicles is possible (9).

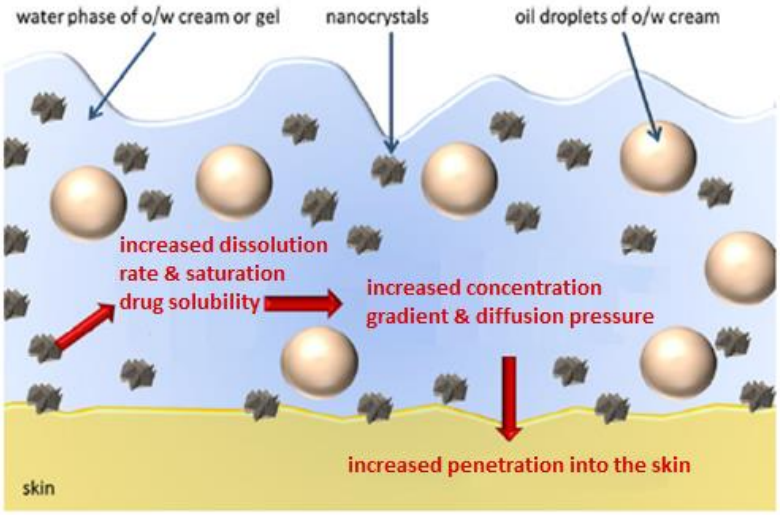

Figure 1: Benefits of nanocrystal formulation in dermal drug administration (11).

1.2.1 Theoretical aspects of increased dissolution rate due to nanonization

Poor solubility (saturation solubility, cs) can be explained by the law of Noyes-Whitney (Equation 1), describing the dissolution velocity dc/dt proportional to the concentration gradient cs – cx (cx – bulk concentration of the drug in the surrounding liquid, which can be described also as Xd/V). Dissolution velocity is also a function of the drug surface area. According to this equation dissolution velocity can be increased by enlarging the drug surface by micronization or nanonization. Further increase in drug solubility and dissolution rate is possible by addition of surfactants (4, 7).

$$\frac{dc}{dt} = \frac{AD}{h}\left(cs - \frac{Xd}{V}\right)$$ [*Equation 1*]

where

d*c*/d*t* = dissolution velocity

Xd = amount dissolved

A = particle surface area

D = diffusion coefficient

V = volume of fluid available for dissolution

c_s = saturation solubility

h = diffusion layer thickness

Saturation solubility is usually a compound specific constant for normally sized particles (particle size > 2 μm), dependent on solvent and temperature (9, 13, 14). However, this constant changes with particles smaller than 2 μm. Ostwald-Freundlich equation (*Equation 2*) is specific for particles smaller than 2 μm, and shows how saturation solubility increases with decreasing particle size. This indicates higher saturation concentration on the nanoparticle surface compared to larger particles surface (13, 15).

$$ln\left(\frac{S}{S0}\right) = \frac{2M\gamma}{\rho rRT} \text{ or } \quad S = S\infty\, exp\left(\frac{2\gamma M}{r\rho RT}\right)$$ [*Equation 2*]

where

S = saturation solubility of nanosized drug

S^{∞} = saturation solubility of an infinitely large crystal (bulk material)

γ = particle-medium interfacial tension

M = compound molecular weight

r = particle radius

ρ = particle density

R = gas constant

T = temperature

1.3 Nanosuspension preparation methods

Nanosuspensions can be prepared by two basic approaches: “bottom-up” and “top-down”. “Top-down” approach is by far more popular and most common technique to produce nanocrystals. “Top-down” processes are based on breaking larger micron-sized particles into nano-sized particles by different wet milling techniques such as media milling, microfluidization, high pressure homogenization (1, 3, 4, 13). “Bottom-up” approach is based on formation of nanocrystals from dissolved drug molecules i.e. by controlled precipitation/crystallization (4). Furthermore, a combination of different methods can also be used (6, 11). The main difference in the procedure of nanocrystal production for oral, intravenous or dermal application is the selection of stabilizers (8, 9).

1.3.1 “Top down” methods

High pressure homogenization

The high pressure homogenization is a high energy process. It involves forcing the drug suspension under high pressure through a small gap. This process causes cavitation and particle collision, which produce forces to break drug particles to smaller sizes (2, 16). It is a relatively fast method, applicable also for production of aqueous-free nanosuspensions. However, it has some disadvantages including high energy consumption, which can generate an amorphous state of drug (2, 17).

Pearl milling

An alternative low energy method to high pressure homogenisation is wet milling technique i.e. pearl milling. Most of the nanocrystal products on the market today are produced by this technology (2, 11). The milling chamber is loaded with the drug, dispersed in a liquid medium containing the stabilizer, and filled with milling pearls (beads), namely milling medium. The drug dispersion and beads rotate during milling. High energy and shear forces are generated which provide the energy needed to reduce the drug particle size. There are two different variations of wet milling: (1) the milling medium is moved by an agitator or (2) the milling chamber is moved, causing the movement of milling medium and generating shear forces (18). Critical parameters to achieve optimal pearl milled product are drug

amount, number and size of the milling pearls, milling speed, milling time and temperature. The amount of the drug in the milling chamber is usually between 2 to 30 percent of the total weight of the suspension. Size of milling pearls is between 0.5 and 1.0 mm. The application of smaller beads usually results in bigger particle size reduction. Nanocrystals can be produced either using low milling speeds (80-90 rpm) and long milling times (1-5 days) or conversely high milling speeds (1800-4800 rpm) and short milling times (30-60 min) (3).

Two completely different processes occur during wet milling, namely diminishing of the drug particles due to the particle fragmentations and particle growth due to the interparticle collisions (3). The occurrence of these two processes depends on the process parameters e.g., milling time, hardness of the drug, viscosity of the drug suspension, temperature, energy input, size of the milling media and stabilizer concentration used (19). After a certain milling time particles have a constant average size and further milling may result in increased average particle size (3).

The benefit of wet milling is that poorly soluble drugs can be easily converted into nanosuspensions either in aqueous or nonaqueous media and the process can be easily scaled-up with little batch to batch variations. The drawbacks of this method, especially at longer milling times, include increased risk of contamination of the final product caused by the erosion of milling pearls, unwanted drug degradation, stability problems and high costs. To avoid the product contamination, milling pearls should be made of highly resistant materials, such as polystyrene resins, zirconium oxide or glass. The control of milling temperature is required, especially when dealing with thermolabile drugs and drugs with low melting points (3, 13). Formation of amorphous regions in drug particles can occur during milling due to the properties of an individual drug, stabilizer and process parameters. Amorphous forms of the product are usually undesirable due to the limited stability and stabilization problems (3).

1.3.2 “Bottom up” methods

Precipitation

The drug is firstly dissolved in an organic solvent and the obtained solution is then added to a non-solvent. The drug molecules precipitate and nanosized drug particles are formed. This method is simple and low cost, but has some drawbacks, namely the use of organic solvents which may result in solvent residues, drug particles tend to grow in the final product, and

many poorly soluble drugs are poorly soluble not only in aqueous, but also in organic media (7, 13). The precipitation is the most widely used "bottom-up" technology, but there are also other methods e.g., spray drying, freeze-drying, high gravity controlled precipitation technology, sonocrystallization, confined impinging liquid jet precipitation, multi-inlet vortex mixing (2).

Spray drying

An alternative "bottom-up" method to precipitation is spray drying, the method where drug solution droplets are sprayed in a special container and dried by hot air. The dry nanosized drug particles can be therefore directly produced (18).

1.3.3 Combination technologies

The combination technologies involve "bottom-up" and "top-down" methods. They can be adjusted to drug properties. Combination of methods improves particle size reduction effectiveness and accelerates nanosuspension production, which leads to more stable nanosuspension with very small drug particles (11, 19). The most frequently reported combination technology is drug precipitation followed by high pressure homogenization (6, 17, 18, 20).

1.4 Stabilization of nanosuspensions

After particle size reduction some interfacial free energy is left over, therefore thermodynamically unstable nanosuspension is formed. Attractive van der Waals forces between dispersed particles become important (4). Consequently, physical instability may occur, resulting in particle agglomeration, aggregation, sedimentation, flotation, caking or crystal growth (9). Ostwald ripening causes crystal growth and agglomeration. It usually appears during storage because of the differences in crystal size and temperature changes resulting in changes in particle solubility (1, 7). To overcome the attractive interactions and prevent unwanted processes, repulsive forces should prevail in the dispersed system (4).

There are two common approaches to achieve sufficient repulsive forces, namely steric and electrostatic approach. An appropriate selection of stabilizer is required. In most cases a combination of stabilizers is preferred, since it enhances long-term stability (1, 4). Non-ionic surfactants and polymers are used as steric stabilizers. They adsorb onto the drug particle surface and make a barrier, which prevents particles to get too close to each other and thus

block van der Waals' attractive forces between particles (3). Electrostatic stabilization is obtained by adsorbing charged molecules, either ionic surfactants or charged polymers, onto the particle surface. Electrostatic repulsion between the particles prevails over the attractive forces (1, 4). Hydroxypropyl cellulose (HPC), hydroxypropyl methylcellulose (HPMC), polyvinyl pyrrolidone (PVP) and poloxamers (poloxamer 188 and poloxamer 407) are commonly used as steric stabilizers (1). Polymer chain of steric stabilizer should be long enough (5.000-25.000 g/mol) to provide a sufficient steric protective layer. Since steric stabilization is sensitive to the temperature fluctuations, a problem could occur if a product needs to be sterile (3). Electrostatic stabilizers can be anionic (sodium lauryl sulfate) or cationic. However, cationic stabilizers are not used in oral formulations because of their antiseptic effect, however they can enhance adhesiveness of nanocrystals in dermal application (4, 19). In addition, electrostatic stabilizers can enhance wetting and dispersion of very hydrophobic drug particles in aqueous medium (4).

Stability of the nanosized drug particles depends on the type of stabilizer used, since it affects the final particle size and formulation stability. The selection of stabilizer depends on many different factors, such as surface energy, hydrophobicity, solubility, viscosity and the functional groups of the drug. Since every drug has different characteristics it demands a different optimal stabilizer. Even preparation method used can affect the selection of the optimal stabilizer. Even though many different factors should be considered when selecting a stabilizer, the most important parameter seems to be the affinity of the stabilizer for the particle surface (3). Poorly soluble drugs are usually hydrophobic, therefore a stabilizer should have some hydrophobic properties to adsorb strong enough to the particle surface (5). High viscosity dispersion media can also contribute to nanosuspension stability, due to decreased diffusion velocity. The amount of stabilizer is crucial for the formulation stability. If the amount of stabilizer is too low, particles tend to aggregate. In case the amount of stabilizer is too high, increased drug solubility due to formation of micelles can occur, resulting in accelerated Ostwald ripening (3, 13). Finally, the drug delivery route should also be taken into account, when selecting an optimal stabilizer (9).

1.5 Characterization of nanosuspensions

Nanosuspensions can be evaluated in a similar way as conventional suspensions i.e. determination of particle size, appearance, colour, odour, and assay of impurities (17).

Appropriate tests for assurance of the quality of nanosuspensions are selected based on the performance required. Methods for characterization of nanosized particle formulations can be classified in two groups. In the first group are the methods used for characterization of dispersed particles e.g., particle size, surface charge (zeta potential), particle crystallinity and dissolution. The methods in the second group measure bulk formulation properties, such as viscosity (4).

1.5.1 Average particle size and particle size distribution

Photon correlation spectroscopy

The most basic property of nanosized particles is their average size and particle size distribution. Particle size may affect other particle characteristics, such as dissolution rate, saturation solubility and physical stability (13). There are many methods available for particle size and size distribution measurement e.g., photon correlation spectroscopy (PCS) and laser diffractomety. PCS, also known as dynamic light scattering, is rapid method for determination of particle size within the range of 0.02 – 3 μm (2). Nanosized particles exhibit random movements due to Brownian motion, which is related to their particle size. Small particles have a higher diffusion velocity and thus move faster. Particle dispersions are illuminated with a laser light and PCS measures time dependent intensity fluctuations of the scattered light (9, 18). This method determines the average particle size and polydispersity index (PI) of the sample (2). PI expresses the width of the particle size distribution. The PI value is 0 in case particles are monodisperse. Samples with narrow particle size distribution have PI values between 0.1 – 0.2. Samples with the PI values above 0.5 have very broad particle size distribution (18).

Laser diffractometry

A better method than PCS to determine presence of any micrometer sized particles in the sample is static light scattering, also known as laser diffractometry (LD). The method is fast and suitable for analysis of large numbers of samples. It can be used for particle size in range 0.02 – 2000 μm. Since this method measures the size of all particles in the sample at the same time, it should be calibrated with a more accurate method (2, 8). It gives information about mean particle size, along with the D_{10}, D_{50} and D_{90} values (i.e. D_{90} value means that 90% of the particles by volume are below the given size) (4, 18).

1.5.2 Surface charge

Another fundamental characteristic of nanosized particles which is important for physical stability of colloidal systems is surface charge. It is assessed through measurements of the zeta potential (ZP). ZP is the potential at the hydrodynamic shear plane and can be determined from the electrophoretic mobility of the particles, which is then transformed into ZP. The mobility of particles depends on the effective charge on the particle surface. Surface charges can appear from ionization of the particle surface functional groups or adsorption of ions from dispersion medium e.g., ionised surfactant molecules onto the surface. Surface charge depends also on electrolyte concentration in dispersion medium. ZP around -20 mV provide only short-term stability, values below -30 mV gives good and below -60 mV excellent stability (8). However, if only electrostatic stabilizer is used, a minimum zeta potential of ±30 mV is required to stabilize nanosuspension (21).

1.5.3 Particle morphology

Instruments used for microscopical observation of nanosuspensions are optical light microscopes, scanning electron microscopes, transmission electron microscopes and atomic force microscopes. Selection of a proper instrument is based on the size range of particles, instrument magnification and resolution. An optical microscope is quite often used for routine investigations of prepared formulations, since it is more affordable and easier to operate and maintain than electron microscopes. However, its applicability is limited due to low magnification and resolution (18).

Scanning electron microscopy (SEM) is a type of electron microscopy which creates images of the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with atoms in the sample, producing various signals which can be detected and provide information about surface topography, crystalline structure, chemical composition and electrical behaviour (conductivity). By SEM an ultimate resolution of 1 nm can be achieved (9, 22).

Transmission electron microscopy (TEM) is a type of electron microscopy where an electron beam is transmitted through a thin sample (100 nm or less). Transmitted electrons interact with the sample and create an image. It can provide information about crystal defects such as dislocations, stacking faults, precipitates and interfaces. TEM enables resolutions reaching atomic scale (~0.1 nm) (23).

Main difference between both types of the electron microscopy is that TEM requires thin samples. Thus, more complex sample preparation is required, which is destructive for the sample (22).

1.6 Selection of drug and stabilizers for nanosuspension preparation

1.6.1 Dexamethasone

Dexamethasone with IUPAC name 9α-fluoro-16α-methylprednisolone (Figure 2) is a well-known drug and its administration is quite wide. It is a potent long-acting corticosteroid with negligible sodium retaining effect (24). It is commonly used as an anti-inflammatory drug, an immunosuppressive, to treat allergies and adrenal cortex insufficiencies, to induce parturition and alleviate stress (25). In dermatology it is used to relieve chronic skin diseases. Its therapeutic concentrations are between 0.01% and 0.05% in ointments, creams and solutions (26). Dexamethasone is nearly insoluble in water (89.0 mg/L at 25°C) (27).

It dissolves in ethanol, 96% (V/V) in weight ratio 1:42 and in propylene glycol in weight ratio 1:100. Dexamethasone is stable in acidic conditions (optimal pH 3.0 – 3.5). In basic conditions it decomposes (26). Dexamethasone has a melting point of 262-264°C and logP value of 1.9 (27).

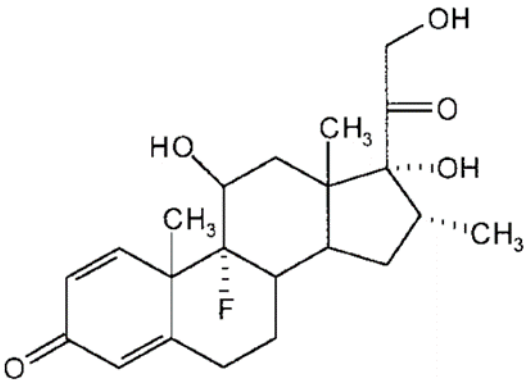

Figure 2: Chemical structure of dexamethasone (25).

According to the literature review, the size of dexamethasone particles has been already reduced to nanodimensions. However, there was no report found about dexamethasone nanocrystal/nanosuspension preparation with a pearl milling method.

High pressure homogenization was used as a "top-down" method to produce dexamethasone nanocrystals with 2.5% of the drug, stabilized with 4% of poloxamer 188. The study showed

enhanced rate and extent of ophthalmic drug absorption as well as the intensity and duration of drug action (28).

Freeze drying as a “bottom up” method for preparation of dexamethasone nanocrystals was used. The method comprising the freeze-drying a frozen sample of mixture of organic solvent, a drug and an ointment base. Freeze-dried sample was afterwards dispersed in water, resulting in formation of nanoparticle aqueous dispersion. Particles of active ingredient are covered with ointment base what consequently inhibits growth of nanocrystals. After production and during storage an average crystal size of approximately 100 nm was observed (29).Spray drying as another “bottom up” technique was used to produce dexamethasone nanocrystals. Mean particle size was approximately 800-1300 nm, depending on the production batch. Particle size was controlled by selecting the mesh aperture size and no stabilizer was added to the formulation (30).

According to the literature data, poloxamer 188, poloxamer 407, hydroxypropyl methylcellulose (HPMC) and vitamin E TPGS were selected as stabilizers for production of dexamethasone nanosuspensions. As reported in several studies, poloxamer 188 has been confirmed to be one of the best investigated nanosuspension stabilizers (1). Poloxamer 407 has already been used for stabilization of dexamethasone (31). HPMC was chosen due to its viscous properties (1). Due to remarkably good stabilization of other drugs vitamin E TPGS (32) was chosen to be used alone and in combinations with previously mentioned stabilizers.

1.6.2 Poloxamer 188 and poloxamer 407

Poloxamer 188 and poloxamer 407 are synthetic copolymers of ethylene oxide and propylene oxide. Figure 3 shows general chemical structure of poloxamers. In poloxamer 188 *block a* has a value of 80 and *block b* 27, while in poloxamer 407 *block a* has a value of 101 and *block b* 56. Both are white, coarse-grained powders with a waxy consistency. They are solid materials at room temperature, soluble in water, as well in polar and non-polar organic solvents. Poloxamer 188 is used as a wetting agent, as emulsifier and solubilizer. It improves solubility, absorption and bioavailability of poorly soluble drugs. It is known for its nontoxic and non-irritant properties, therefore it is applied in liquid dosage forms for parenteral, oral or dermal application. Poloxamer 407 is used as thickening agent and gel former, a co-emulsifier, a consistency enhancer in dermal products and as a solubilizer for some poorly soluble drugs. Due to its ability to affect the formulation viscosity it is often

used as a stabilizer for topically administered products. Poloxamer 407 is used in toothpastes, gargles and mouthwashes as well (33). Finally, the comparison of stabilization properties of poloxamer 188 and poloxamer 407 reveals that poloxamer 188 is better stabilizer. It was able to stabilize majority of the investigated model compounds. The better performance compared to poloxamer 407 is suggested to be due to its lower molecular weight (32).

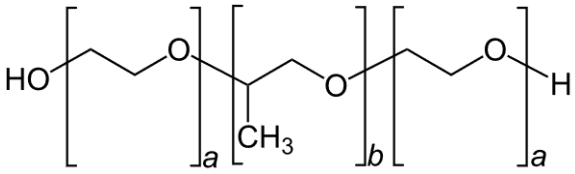

Figure 3: Chemical structure of poloxamers (33).

1.6.3 Hydroxypropyl methylcellulose (HPMC)

Cellulose derivative, hydroxypropyl methylcellulose (Figure 4) is a steric stabilizer with high molecular weight. It is an effective steric stabilizer, since it increases viscosity of the nanosuspension and thus minimizes particle mobility and reduces direct contact of the nanosized drug particles by covering their surface (17).

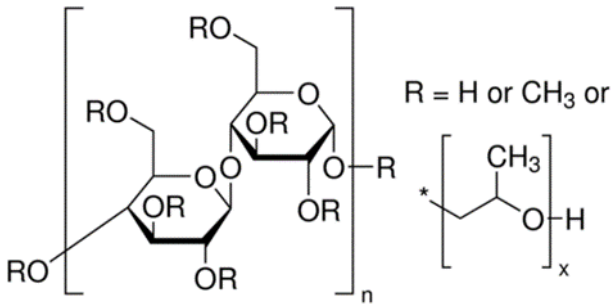

Figure 4: Chemical structure of HPMC (34).

1.6.4 Vitamin E TPGS

D-α-tocopheryl polyethylene glycol 1000 succinate or vitamin E TPGS (Figure 5) is a non-ionic water soluble derivative of vitamin E, which is formed by esterification of vitamin E succinate with polyethylene glycol 1000. Molecule has both lipophilic and hydrophilic properties, so the molecule is amphiphilic. It enhances solubility and hence the bioavailability of many poorly soluble drugs. Water dispersion of vitamin E TPGS has relatively low viscosity and reduces milling time due to high attrition rate of the beads (1, 35). In literature it is described as a solubilizer, permeability enhancer and stabilizer. Molecule has antioxidant properties. It may be used as a surfactant and for preparation of

biodegradable polymers (36). According to the literature, vitamin E TPGS was shown to have a good stabilizing performance in comparison with other stabilizers (32). However, it was observed that vitamin E TPGS as a stabilizer alone induces crystal growth, indicating the need of additional stabilizer (1).

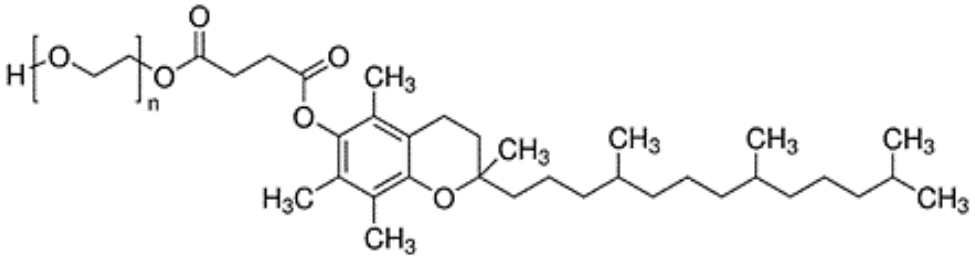

Figure 5: Chemical structure of vitamin E TPGS i.e. D-α-tocopheryl polyethylene glycol 1000 succinate (36).

1.7 Preservatives

Preservatives can be added to nanosuspension to prevent microbial growth. In general preservation can be achieved either by chemical or physical approach. Physical is refrigeration and drying. Chemical entails the use of chemical compounds (37). If a product contains water and it is not determined to be a sterile single-unit preparation, it needs to be preserved (38). Adequate preservation of nanosuspensions is important for all administration routs. However, preservation can impair the formulation stability, e.g. causes reduction of absolute value of ZP, dehydration of stabilizer layer, displacement of stabilizer from nanocrystal surface. Effect of preservation on formulation stability can be determined by particle size measurements during physical stability tests. Hydrophobic preservatives have usually a large influence on the formulation stability. The higher is their hydrophobicity, the stronger is their destabilizing effect on nanosuspension (Figure 6). Better adsorption to the particle surface of nanosuspensions can result in a preservative inefficiency (12, 39). According to the literature data, preservatives used in dermal formulations of dexamethasone include (a.) potassium sorbate 0.07% and sorbic acid 0.05%, (b.) sorbic acid 0.1% and potassium sorbate 0.14%, (c.) propylene glycol 20% and (d.) esters of parahydroxybenzoic acid 0.1% (26, 37).

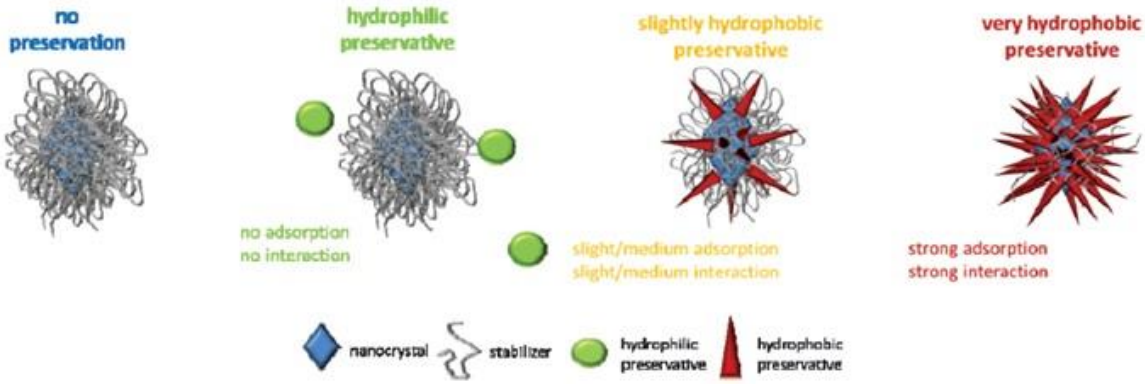

Figure 6: Preservation of nanocrystal formulation by hydrophilic, slightly hydrophobic and very hydrophobic preservative (39).

Propylene glycol and benzyl alcohol belong to the group of aliphatic and aromatic alcohols. Propylene glycol is a very hydrophilic preservative and thus has smaller tendency to adsorb to the surface of the particles (38). Since propylene glycol does not evaporate upon the application of formulation it can improve adhesion of the formulation to the skin (37). Sorbic acid belongs to the group of carbon acids.

Based on the literature data propylene glycol, benzyl alcohol and a combination of sorbic acid and potassium sorbate were chosen as preservatives in our study.

2 OBJECTIVES

The aim of this work is to produce and characterize dexamethasone nanosuspension with the purpose to improve the drug properties. Drug in form of nanosized particles is expected to penetrate better into the skin, because of its improved dissolution properties. The method applied for reduction of the drug particle size will be pearl milling. The characteristics of the particles will be studied as a function of the type of stabilizer used, type of preservative added, storage conditions and time.

Dexamethasone nanosuspensions will be prepared following the common method. The only difference between nanosuspensions will be the choice of stabilizer. Four stabilizers appropriate for dermal administration will be studied alone and in combinations. Particle size reduction efficacy during the milling will be analysed and compared between nanosuspensions. The analysis will be based on particle size and particle size distribution.

Produced nanosuspensions will be analysed in short-term stability test to evaluate their physical stability. This will be performed for 30 days at three different temperatures. Characterization of stored nanosuspensions will be carried out regularly on day 1, day 7, day 14 and day 30 after production. The obtained data will be compared with measurements performed right after production of nanosuspensions (day 0). Characterization results will consist of average particle size, size distribution, zeta potential measurements, laser diffraction measurements and if needed, light microscopy data.

Many of preservatives can destabilize nanosuspensions, therefore, the influence of three different preservatives with different chemical properties on nanosuspension physical stability will be investigated. The selected preservatives will be suitable for dermal administration. The average particle size, size distribution and zeta potential will be measured directly after production of preserved nanosuspensions. Preserved nanosuspensions will take part in a short-term stability test as well. Microbiological stability of nanosuspensions will be tested using agar plates in a time frame of two weeks.

Produced nanosuspensions will be analysed also by UV spectroscopy, aiming to determine the amount of dissolved drug in the aqueous medium.

3 MATERIALS AND METHODS

3.1 Materials

The following materials were used in this study: the drug was micronized dexamethasone (Fagron, USA), stabilizers used were poloxamer 188 (Lutrol F68, BASF, Germany), poloxamer 407 (Lutrol F127, BASF, Germany), vitamin E TPGS (Kolliphor TPGS, Speziol TPGS Pharma, BASF, Germany) and HPMC (Methocel, Colorcon, UK). Nanosuspensions were formulated with freshly prepared double distilled and ultra-purified water (Milli-Q, Millipore GmbH, Germany).

The preservatives used were 1,2-propylene glycol (1,2-Propylenglykol USP, BASF, Germany), sorbic acid >99% purity, Ph.Eur. (Sorbinsäure >99% reinst, Ph.Eur., ROTH, Germany), potassium sorbate (Sigma-Aldrich, Germany), citric acid (Citronensäure, Dr. Paul Lohmann, Germany) and benzyl alcohol anhydrous, 99.8% (Sigma-Aldrich, Germany).

3.2 Preparation of dexamethasone nanosuspension

All nanosuspensions were produced by a pearl milling method. They were prepared according to the following procedure. The first step was preparation of dispersions of the drug and stabilizer in an aqueous media (1). The weight ratio of drug to stabilizer is according to the literature data usually from 20:1 to 2:1 (3), therefore, we have chosen the drug concentration of 2.0% (w/w) and stabilizer concentration of 0.8% (w/w) with respect to the total weight of dispersion. Ideally, the preservative is added prior the milling process. However, to prevent displacement of the stabilizer from the particle surface and impairing the diminution efficacy (12, 39), it was added after the milling. The drug, stabilizer and dispersion medium (purified water) were weighed prior milling to produce in total 50 g of dispersion with preservative (10.0% (w/w)) (38), which was added after wet milling. The ingredients were mixed with IKA Ultra-turrax T25 (IKA, Germany) at 10,000 rpm for 1 min to produce a macrosuspension. A sample of macrosuspension (~0.5 g) was stored for further analysis to determine the average size of unmilled drug particles. The rest of macrosuspension was put into the milling chamber of Dyno Mill Typ KDL A (Willy A. Bachofen AG Maschinenfabrik, Switzerland) (Figure 7), together with 90 g of zirconium oxide beads (Φ = 0.3 mm, TOSOH, Japan).

Figure 7: Left - Dyno Mill Typ KDL A, glassy milling chamber was adjusted to the stainless steel circle part just prior milling; right – glassy milling chamber with forced cooling water flow as a cooling system and pressure optimizer/releaser.

The milling was performed at speed of 3,200 rpm and time of the milling was 30 min (1). Sampling was done at regular time intervals to see how particle size changes during the milling. The samples were withdrawn with a syringe and a needle after 5, 10, 15 and 20 min of milling. After 30 min of milling the prepared nanosuspension was separated from the beads and transferred to a vial. At this point a preservative was added to a half of the product. As a result there were seven different samples from one batch, all in vials, sealed with parafilm and prepared for further analysis (Figure 8).

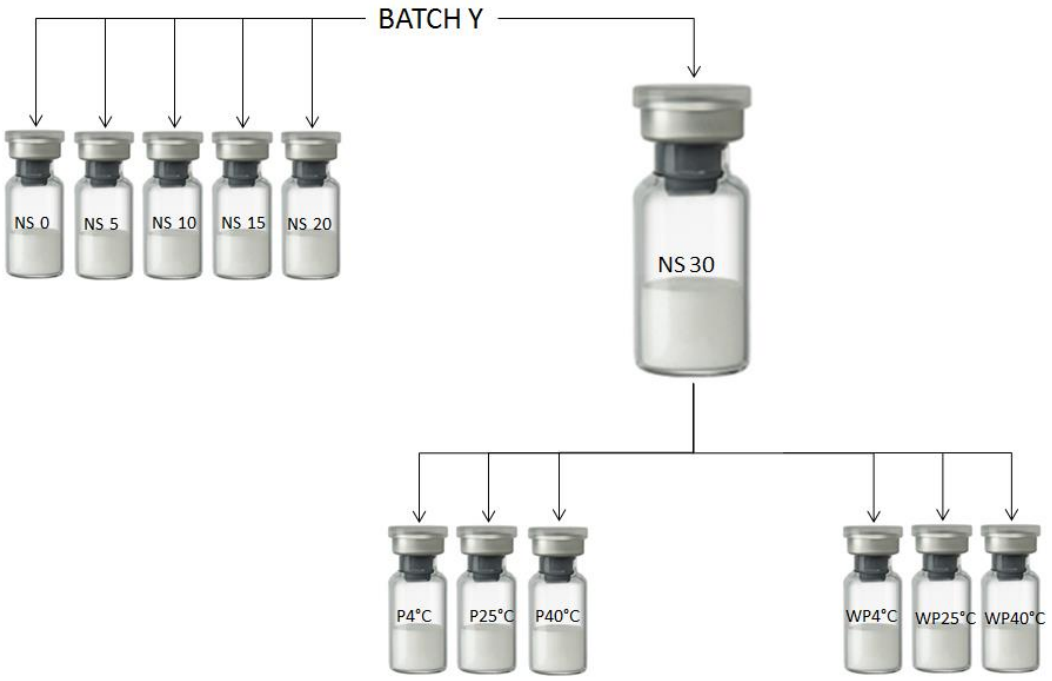

Figure 8: Samples of nanosuspensions from one batch (above: NS stands for nanosuspension, numbers define the time of milling; below: P means preservative was added, WP means without preservative, degrees define the temperatures samples were stored at).

The stabilizers and preservatives used in prepared nanosuspension formulations are shown in Table I. Firstly, four different stabilizers were tested: poloxamer 188, poloxamer 407, vitamin E TPGS and HMPC. All the samples contained the same amount of the drug, water and stabilizer and were prepared according to the previously described procedure. The only exceptions were two batches, one stabilized with HPMC and one with vitamin E TPGS. In the first one a lower amount of HPMC was used (0.1%). In case of nanosuspensions stabilized with Vitamin E TPGS the amount of stabilizer was unchanged, but it required longer time to dissolve in water (~60 min) before a macrosuspension could have been prepared.

Three different preservatives were tested: 1,2-propylene glycol, benzyl alcohol and a combination of sorbic acid and potassium sorbate. Nanosuspensions were preserved with 1,2-propylene glycol, which was tested in concentration range 10-20% (w/w) (37). Other two preservatives were studied only in nanosuspensions stabilized with poloxamer 407. A preservative mixture of sorbic acid and potassium sorbate was prepared from 0.1% (w/w)

sorbic acid, 0.14% (w/w) potassium sorbate and citric acid was added additionally to adjust pH. The latter was checked with pH Meter/mV meter PB-11 (Sartorius, Germany). pH should be below 4.74 to keep sorbic acid protonated and maintain preservative efficacy (38).

Table I: Stabilizers and preservatives used in nanosuspension formulations.

Batch	Stabilizer used	Amount (%)	Preservative used
I	Poloxamer 188	0.4	1,2-Propylene glycol
II	Poloxamer 407	0.4	1,2-Propylene glycol
III	HPMC	0.4	1,2-Propylene glycol
IV	Vitamin E TPGS	0.4	1,2-Propylene glycol
V	Vitamin E TPGS + Poloxamer 188	0.2 + 0.2	1,2-Propylene glycol
VI	Vitamin E TPGS + Poloxamer 407	0.2 + 0.2	1,2-Propylene glycol
VII	Vitamin E TPGS + HPMC	0.2 + 0.2	1,2-Propylene glycol
VIII	Poloxamer 407	0.4	1,2-Propylene glycol, benzyl alcohol, sorbic acid + potassium sorbate
IX	HPMC	0.1	1,2-Propylene glycol, benzyl alcohol, sorbic acid + potassium sorbate
X	Poloxamer 407	0.4	1,2-Propylene glycol, benzyl alcohol, sorbic acid + potassium sorbate

3.3 Characterization of dexamethasone nanosuspensions and nanocrystals

3.3.1 Average particle size and particle size distribution

The average particle size and PI of produced nanosuspension samples were measured with Malvern Zetasizer Nano ZS (Malvern instruments, UK). Before the analysis all samples were diluted with saturated aqueous dexamethasone solution (stock solution), which was prepared at least one day prior measurements. The stock solution was prepared according to the following procedure: approximately 120 mg of the drug was added to 1 L of purified water, stirred on a magnetic stirrer overnight and filtered through a 5.0 μm cellulose acetate filter (Sartorius, Göttingen, Germany) before use to remove any unsolved drug particles. Dilution of nanosuspension in water would result in partial dissolution of drug nanocrystals

and consequently resulting in misleading measurements. Nanocrystals did not dissolve in stock solution since it was already saturated with the drug. Samples were diluted 1000-times with stock solution. All samples were analysed at 25°C in ten replicates.

3.3.2 Laser diffractometry

Since the Zetasizer Nano ZS cannot detect larger particles, the particle size was analysed with LD using a Malvern Mastersizer 2000 with a Hydro 2000S wet dispersion unit (Malvern instruments, UK). Sonication before the measurement was not carried out to prevent the destruction of any aggregates in the sample. In case aggregates were present another measurement after sonication was made. Samples to be analysed with LD were diluted when they were added to the stock solution inside the laser diffractometer. When the sample obscurity was high enough the machine gave a notification to start. The results were analysed using Mie theory (particle refractive index of 1.591 and imaginary refractive index of 0.01 were used).

3.3.3 Zeta potential measurement

The surface charge of drug particles was measured straightaway after nanosuspension preparation using laser Doppler anemometry (Zetasizer Nano ZS). ZP was measured using the same diluted sample as was used for the particle size analysis using the same device. ZP measurements were performed with a disposable folded capillary cell with electrodes on both sides (Figure 9). The Helmholtz-Smoluchowski equation built in the device software was used for data evaluation. All samples were analysed at 25°C in triplicates.

Figure 9: Disposable folded capillary cell. It has negative and positive electrode connected with a capillary, which is filled with a sample (40).

3.3.4 Light microscopy

Light microscopy was used to identify the morphology of the produced nanocrystals and to confirm results obtained by the LD analysis. A light microscope (Ortophlan, Leitz, Germany) equipped with a CMEX-1 digital camera (Euromex microscopes, Netherlands) was used. Any instability resulting in particle aggregation can be clearly visible under the light microscope. Within one day after nanosuspension preparation the samples were analysed under light microscope using polarized and non-polarized light at magnification of up to 1000-fold.

3.4 Short-term physical stability test

To examine physical stability of nanosuspensions and to identify the most effective stabilizer, each batch of nanosuspension was divided into six vials and stored at different storage conditions (protected from light at 4°C, 25°C and 40°C) (Figure 8). Short-term stability test lasted 30 days. Characterizations were carried out regularly on the day of production (day 0), day 1, day 7, day 14 and day 30. Average particle size, particle size distribution, ZP, LD measurements and, if needed, light microscopy, were performed.

3.5 Microbiological tests

To evaluate, if the applied concentration of preservative was effective against the bacteria, agar plates were streaked with a sample of nanosuspension a few hours after its production. The streaking was performed aseptically in a laminar air flow chamber. Streaking of agar plates was done with pure 1,2-propylene glycol (negative control), with nanosuspension without preservative (positive control) and with nanosuspension with added preservative (test sample). Afterwards agar plates were stored in Heraeus heating and drying oven (Heraeus, Germany) at 32.5°C with a maximum time of two weeks.

Agar plates were checked every day after the streaking. In case any microbial growth was observed, notes were made and pictures were taken. Agar plate was put back to incubator until the end of the testing period or until intense growth of colonies was observed.

3.6 UV spectroscopy

This method was used to determine the amount of the drug dissolved in the nanosuspension. Prior to the UV spectroscopy samples of nanosuspensions were centrifuged using Heraeus Biofuge Stratos Centrifuge (Heraeus, Germany) at 17,000 rpm for 3 h at 25°C. At least 1

mL of supernatant of centrifuged nanosuspension was needed for each measurement. Some samples were not measured, due to the lack of sample quantity, since some nanosuspensions were produced in smaller amounts or they were already spent for other analysis. There were issues as well while measuring samples stabilized with vitamin E TPGS, probably due to its lipophilicity and its influence on sample absorbance. The samples were analysed at fixed wavelength of 242 nm. Purified water was used as a blank. Absorption spectrum of 0.8% (w/w) aqueous solution of stabilizer was also measured to exclude presence of any stabilizer peaks at analytical wavelength. All samples were analysed in duplicate.

3.7 Statistical analyses

Particle size data are reported as arithmetic means ± standard deviation of ten measurements. The data were obtained using Zetasizer Software, which provides excellent ensemble statistics for an average particle size (by intensity), the standard deviation of measurements, the relative standard deviation of measurements, the minimum, the maximum and the mean of a selected number of measurements, average polydispersity index, and a moderately peak-resolved distribution by mathematical inversion.

Zeta potentials are presented as arithmetic means of three measurements provided by Zetasizer Software.

4 RESULTS AND DISCUSSION

4.1 Particle size reduction as a function of milling time

The selection of an optimal stabilizer is crucial for nanosuspension stability, since every stabilizer differs in its ability to preserve the particle size in nanosuspension. In the current study four different stabilizers were evaluated: poloxamer 188, poloxamer 407, HPMC and vitamin E TPGS. Results of particle size reduction during the milling process are shown in Table II.

Table II: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions. Samples were withdrawn after 0, 5, 10, 15, 20 and 30 min of milling. Results demonstrated represent the average of ten measurements.

Stabilizer/Time		**0 min**	**5 min**	**10 min**	**15 min**	**20 min**	**30 min**
P188	d (nm)	3485	610.6	583.0	555.7	554.0	658.2
	PI	0.965	0.485	0.469	0.437	0.431	0.461
P407	d (nm)	3597	706.1	452.2	393.1	385.7	334.7
	PI	1.000	0.548	0.356	0.385	0.345	0.362
HPMC 0.8%	d (nm)	4121	1143	975.4	855.0	846.0	618.5
	PI	1.000	0.788	0.562	0.533	0.573	0.317
TPGS	d (nm)	1414	835.9	640.7	496.4	437.2	432.4
	PI	0.760	0.706	0.566	0.440	0.382	0.306
TPGS + P188	d (nm)	3265	605.1	412.3	393.4	375.8	377.8
	PI	0.918	0.251	0.379	0.341	0.301	0.241
TPGS + P407	d (nm)	2842	519.6	370.9	330.8	312.7	334.0
	PI	0.983	0.394	0.297	0.259	0.247	0.297
TPGS + HPMC	d (nm)	4299	1079	929.3	832.2	745.31	647.8
	PI	1.000	0.461	0.556	0.259	0.244	0.353
HPMC 0.1%	d (nm)	2902	1212	786.4	742.6	620.4	534.7
	PI	1.000	0.598	0.560	0.496	0.363	0.479

The most effective particle size reduction was observed in case of nanosuspension that was stabilized with a combination of vitamin E TPGS and poloxamer 407. The smallest average particle size and most homogenous particle size distribution were demonstrated after milling, indicating that particle size reduction was slightly more effective when a combination of stabilizers was used. When only poloxamer 407 was used as stabilizer similar average particle size was achieved, but the particle size distribution was wider than with a combination of poloxamer 407 and vitamin E TPGS. The combination of vitamin E TPGS with poloxamer 188 improved particle size reduction compared to poloxamer 188 alone i.e. the average particle size was smaller and more homogenous. Milling of dispersion stabilized

with poloxamer 188 for 15 to 20 min did not cause any significant change in the particle size. However after 30 min there was a significant increase in average particle size. According to the literature this happens quite often (3). Another interesting observation happened when using HPMC as a stabilizer. In contrast to dispersions stabilized with poloxamers, dispersions stabilized with HPMC required longer milling times, due to the higher viscosity of dispersed medium which made milling less effective. Another disadvantage associated with increased sample viscosity was difficult separation of produced nanosuspension from the milling beads. A significant amount of nanosuspension adhered to the beads and milling chamber, therefore less final product was obtained. Decrease in the percentage of HPMC used did not reduce the problems associated with increased sample viscosity. On top of that, the average particle size after 30 min of milling was smaller in case of smaller HPMC concentration compared to the higher percentage of HPMC, which means, higher percentage of HPMC demands longer milling times to achieve the desired particle size. When milling dispersion stabilized with vitamin E TPGS alone for 20 to 30 min average particle size barely changed, however the PI decreased. That is most likely due to smaller influence of stabilizer used on the dispersion viscosity, therefore the beads can achieve higher attrition and milling time can significantly reduce the PI (1). When the nanosuspension was stabilized with a combination of vitamin E TPGS and HPMC milling was the least effective. There was no observable difference in size when it was compared to a nanosuspension stabilized with 0.8% HPMC. So it can be concluded that the addition of vitamin E TPGS to HPMC did not improve the formulation.

4.2 Short-term physical stability of nanosuspensions

The nanosuspensions with and without preservative added were stored at three different temperatures (4°C, 25°C, 40°C) and were analysed during a 30 day period. Storage of samples at low temperatures can enhance drug recrystallization due to decrease in drug solubility, consequently particle size increases. High temperatures may increase the drug solubility, therefore smaller particles dissolve, larger particles remain and the average size of particles in the dispersion increases (8, 12). These processes in nanosuspensions can be clearly observed or not detected at all (8). Characterization of nanosuspensions was performed straight after production (day 0) and after 1, 7, 14 and 30 days.

4.2.1 Average particle size and particle size distribution

Figure 10 shows results of ten measurements for nanosuspension stabilized with poloxamer 188. Average particle size and PI have increased significantly at all storage temperatures within a month. A significant increase in average particle size was observed at 4°C in comparison to other storing conditions. Some fluctuations in average particle size were observed at 40°C most probably due to larger sized particles, which were less stable therefore; they aggregated and were out of the measuring range of Zetasizer. Consequently the measured average particle size decreased resulting in misleading results.

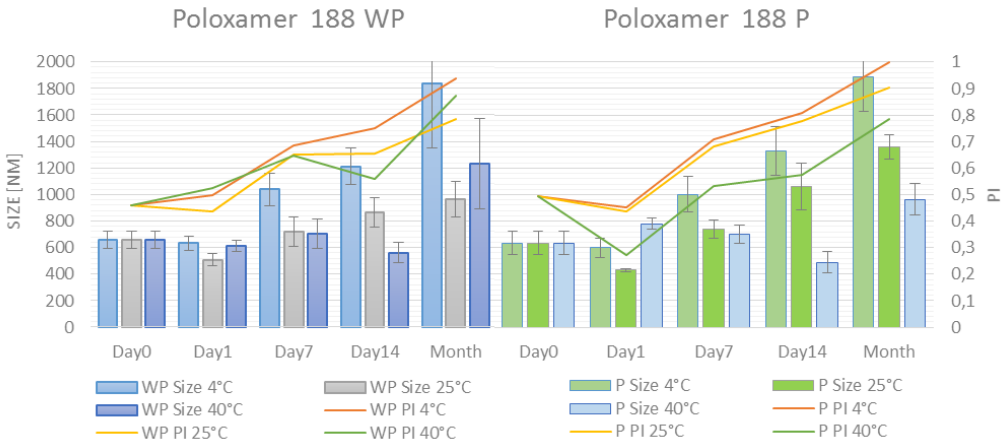

Figure 10: Average particle size and polydispersity index (PI) of dexamethasone nanosuspension stabilized with poloxamer 188 over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

Stabilization of nanosuspension with poloxamer 407 resulted in relatively stable particle size (Figure 11). The average particle size and PI did not significantly increase over 30 days. Furthermore, PI seems to decrease until the end of the test period and a similar tendency of particle size as in the case of nanosuspension stabilized with poloxamer 188 was observed i.e. increase in average particle size at 4°C over the investigated time period. On the other hand, at 25°C average particle size decreased over the time and at 40°C slightly smaller decrease in particle size was observed.

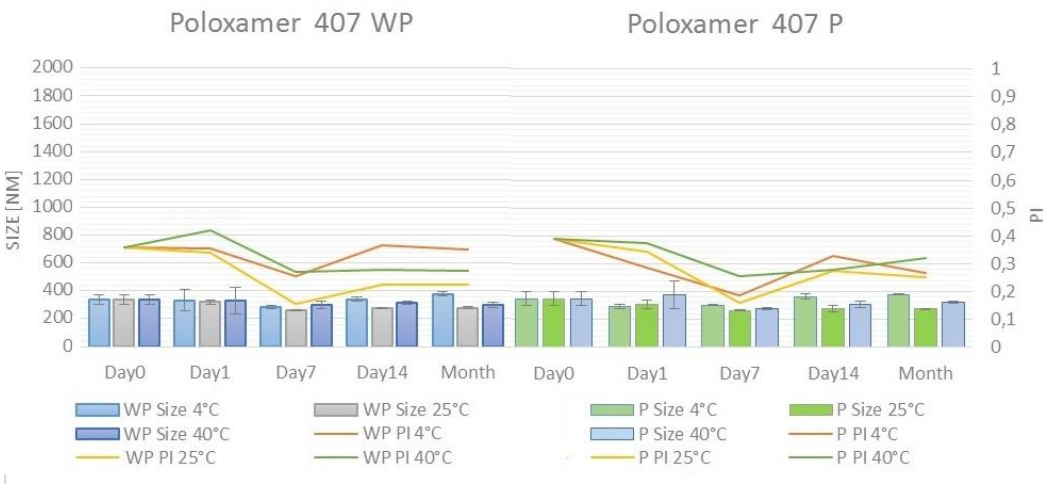

Figure 11: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with poloxamer 407 over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

Nanosuspensions stabilized with 0.8% HPMC did not show any significant increase in average particle size or particle size distribution over the time period of 30 days (Figure 12). This was due to the interaction of the hydrophobic (methoxyl) and hydrophilic (hydroxypropyl) groups of HPMC with the drug particle surface, resulting in good steric stabilization (1). Another factor was increased viscosity of dispersion medium due to HPMC, which impeded the particle dissolution or aggregation.

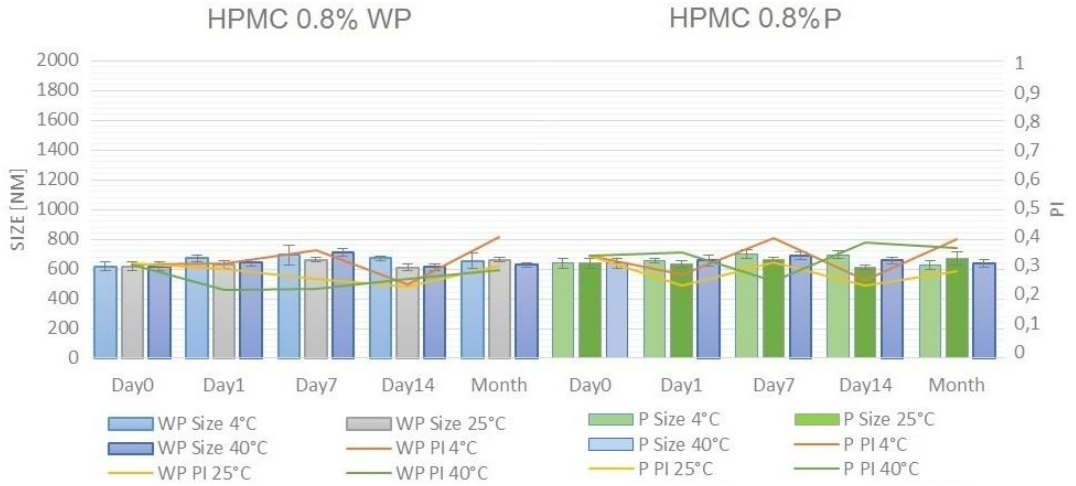

Figure 12: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with HPMC over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

When smaller concentration of HPMC was used as stabilizer, the results were different compared to higher HPMC concentration. Nanosuspension stabilized with 0.1% HPMC exhibited slight particle growth in the short-term stability test, however PI remained stable (Figure 13).

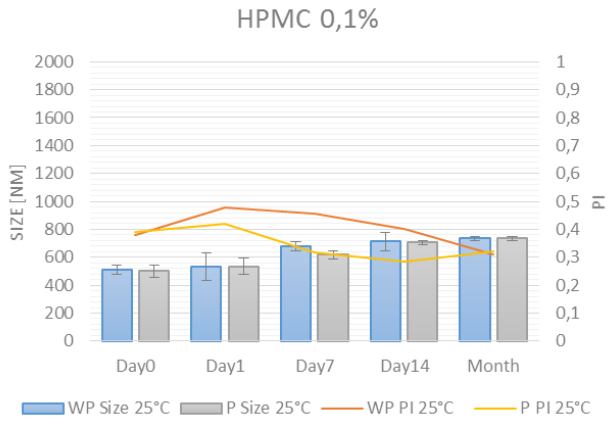

Figure 13: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with 0.1% HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative and P – nanosuspensions with added preservative (P).

In case of nanosuspensions stabilized with vitamin E TPGS the average particle size increased with the storage temperature and storage time (Figure 14). One of the studies published in the literature revealed that the increase in average particle size is due to crystal growth and not due to particle agglomeration (1). LD analysis and light microscopy confirmed the suggested reason, since LD results showed increase in D95 value (Table VII) and light microscopy did not show any difference in sample appearance. There was no significant agglomeration observed after a month of storage. This observation indicates the need for additional stabilizer, since vitamin E TPGS alone cannot stabilize the drug particle size successfully.

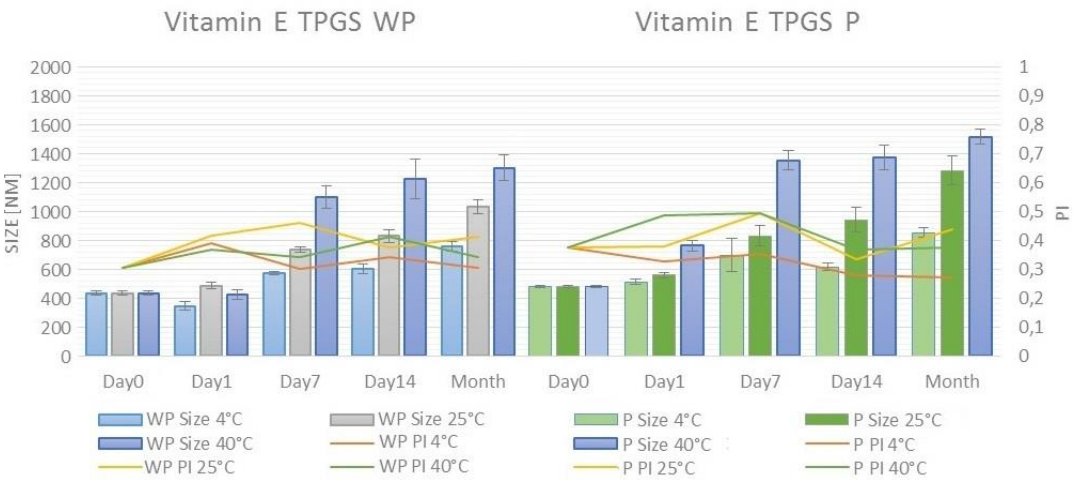

Figure 14: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with vitamin E TPGS over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

In nanosuspensions, stabilized with combination of vitamin E TPGS and poloxamer 188, the particle growth was inhibited to some degree (Figure 15). According to the literature data the combination of vitamin E TPGS and poloxamer 188 can inhibit particle growth (41). However, slight increase in average particle size was demonstrated after day 7 in our study, especially at 25°C and 40°C.

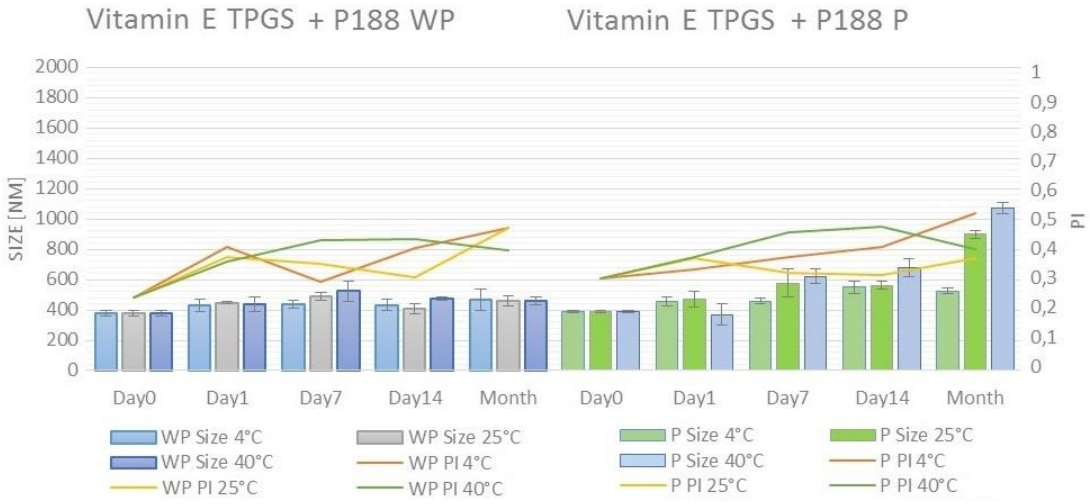

Figure 15: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with a combination of vitamin E TPGS and poloxamer 188 over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

Stabilization of nanosuspension with the combination of vitamin E TPGS and poloxamer 407 resulted in similar results as observed for nanosuspension stabilized with vitamin E TPGS and poloxamer 188, however the degree of particle growth was bigger (Figure 16). The average particle size and PI increased with higher storage temperature.

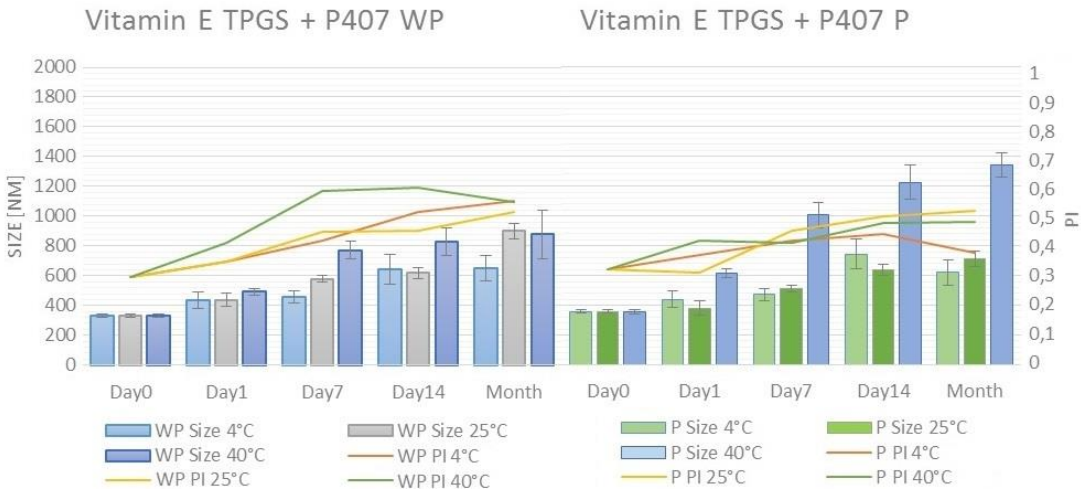

Figure 16: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with a combination of vitamin E TPGS and poloxamer 407 over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

The last, stabilization of nanosuspension with the combination of vitamin E TPGS and HPMC resulted in only small increase in average particle size at all investigated temperatures (Figure 17). Moreover, PIs have decreased slightly with time. Similar results were reported before for combination of vitamin E TPGS and HPMC, which minimally influenced the average particle size compared to other combinations of vitamin E TPGS with poloxamers or vitamin E TPGS alone (1). The main reason are characteristics of HPMC that have been described previously, namely influence on viscosity of dispersion medium and effective steric stabilization.

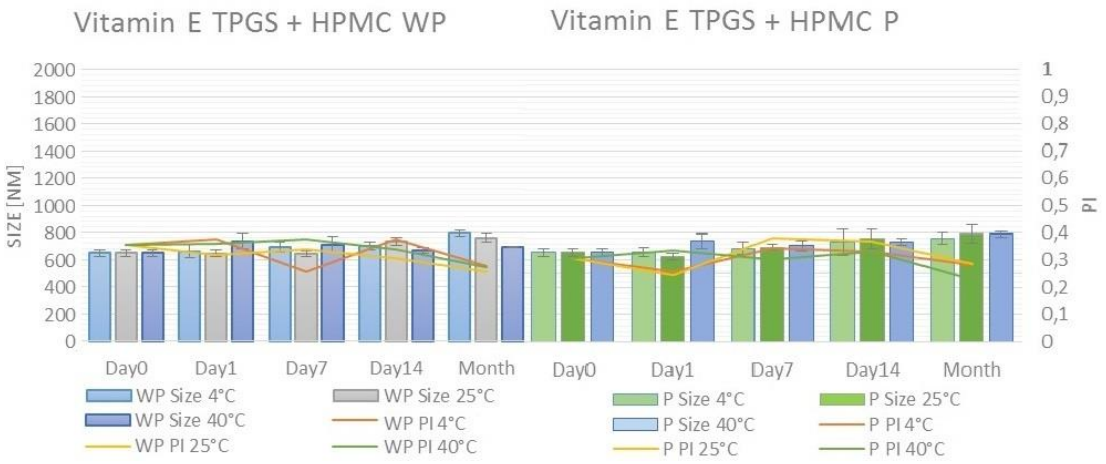

Figure 17: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with a combination of vitamin E TPGS and HPMC over time period of one month (30 days). Left – nanosuspensions without added preservative (WP) and right – nanosuspensions with added preservative (P).

4.2.2 Presence of micrometer-sized particles

LD data of poloxamer 188 stabilized nanosuspensions show, that there were some large microsized particles in non-preserved samples stored at 25°C and in preserved samples stored at 40°C after one month of storage (Table III). The aggregates seem to be stable and difficult to break, since sonication of sample before LD analysis did not break them. The presence of aggregates was afterwards checked and confirmed also with light microscopy. Samples, stored at 4°C, which exhibited some aggregates detected by PCS analysis (Figure 10), did not show any large aggregates with light microscopy test. This could be due to the reason that the LD instrument has a build-in stirrer which can disaggregate loose particle agglomerates. The presence of some aggregates was confirmed also by light microscopy.

Table III: Results of LD analysis of dexamethasone nanosuspensions, stabilized with poloxamer 188 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

P188	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [µm]		D95 [µm]		D95 [µm]	
Day0	4.24	5.85	4.24	5.85	4.24	5.85
Day1	5.07	6.68	6.74	9.75	8.32	9.36
Day7	10.77	3.74	7.08	9.83	10.23	6.32
Day14	10.99	12.62	10.99	10.12	8.93	12.60
Month	9.09	9.78	33.49	12.15	9.95	57.38

The initial size of drug particles in dispersion stabilized with poloxamer 407 was slightly bigger compared to samples stabilized with poloxamer 188. Throughout the test period slow growth of particles occurred (Table IV). Light microscopy confirmed the homogeneity of particle size in the samples, with exception of preserved samples after a month of storage. Taking into the account LD and PCS measurements (Figure 11), it can be concluded, that nanosuspensions exhibited some short-term stability, since average particle size did not change significantly throughout 1 month and there was only a slight increase in LD measurements. However, slight increase in particle size over the time is not considered to impair the dermal use of formulations (8).

Table IV: Results of LD analysis of dexamethasone nanosuspensions, stabilized with poloxamer 407 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

P407	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [µm]		D95 [µm]		D95 [µm]	
Day0	9.63	8.42	9.63	8.42	9.63	8.42
Day1	9.78	18.74	13.47	12.02	18.25	22.23
Day7	21.19	22.24	23.63	21.85	35.90	31.85
Day14	39.41	38.32	34.32	32.78	53.81	50.46
Month	47.67	100.65	68.10	94.19	33.97	34.13

LD data for nanosuspensions stabilized with HPMC are in line with the PCS results. LD data show decrease after day 7 and then again slight increase after a month (Table V).

Table V: Results of LD analysis of dexamethasone nanosuspensions, stabilized with HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

HPMC	4°C		25°C		40°C	
0.8%	**WP**	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [μm]		D95 [μm]		D95 [μm]	
Day0	2.92	3.72	2.92	3.72	2.92	3.72
Day1	1.52	1.65	1.43	1.61	1.32	1.65
Day7	0.52	0.61	0.73	0.71	0.70	0.71
Day14	0.93	0.99	1.03	0.89	0.99	1.48
Month	1.13	1.28	1.50	1.00	1.06	1.74

LD data for nanosuspension stabilized with lower percentage of HPMC slightly fluctuate, but exhibit no big change over the time (Table VI). The data are in line with PCS measurements (Figure 13), which were confirmed also by light microscopy.

Table VI: Results of LD analysis of dexamethasone nanosuspensions, stabilized with 0.1% HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

HPMC	**LD**	
0.10%	**WP**	**P**
	D95 [μm]	
Day0	2.03	1.57
Day1	2.96	1.64
Day7	3.62	2.14
Day14	4.47	5.30
Month	3.22	1.65

LD data confirmed the trend observed with PCS in case of nanosuspensions stabilized with vitamin E TPGS (Table VII, Figure 14) i.e. the higher was the storage temperature, the larger were the particles with time. The particle size was not decreased by sonication. However, the preserved nanosuspension stored at 40°C showed the smallest particle size (Table VII).

Table VII: Results of LD analysis of dexamethasone nanosuspensions, stabilized with vitamin E TPGS over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [μm]		D95 [μm]		D95 [μm]	
Day0	4.87	3.97	4.87	3.97	4.87	3.97
Day1	4.31	2.95	3.06	1.44	14.31	3.10
Day7	6.16	1.75	12.89	12.14	11.31	3.32
Day14	7.44	8.26	9.15	22.30	24.09	3.82
Month	5.38	6.96	13.19	12.57	22.89	3.50

Nanosuspensions, stabilized with a combination of vitamin E TPGS and poloxamer 188, demonstrated some particle growth over the time. The results show larger particles appeared at 40°C what is not completely in line with the PCS results (Table VII, Figure 15). Weak aggregation occurred in preserved samples at 25°C and 40°C after a month, since particle size was decreased by sonication, resulting in LD D95 value up to 12 μm.

Table VII: Results of LD analysis of dexamethasone nanosuspensions, stabilized with vitamin E TPGS and Poloxamer 188 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS	4°C		25°C		40°C	
+ P188	**WP**	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [μm]		D95 [μm]		D95 [μm]	
Day0	4.54	3.03	4.54	3.025	4.54	3.03
Day1	6.23	7.21	7.03	7.11	6.00	11.90
Day7	9.90	12.60	8.06	12.44	10.68	20.3
Day14	7.90	6.90	4.40	8.63	10.70	13.0
Month	9.80	5.60	2.81	15.74	9.82	16.67

LD data for nanosuspension stabilized with vitamin E TPGS and poloxamer 407 showed presence of aggregates, what is not completely in line with PCS results (Table IX, Figure 16). PCS results showed bigger increase in average particle size at 25°C and 40°C, however, average particle size determined by PCS did not increase much in samples stored at 25°C. Large aggregates with size in the range of 5 to 35 μm were outside the measuring range of PCS, so characterization of particles only with PCS gave inaccurate results. Aggregates were also identified by light microscopy.

Table IX: Results of LD analysis of dexamethasone nanosuspensions, stabilized with Vitamin E TPGS and Poloxamer 407 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS	4°C		25°C		40°C	
+ P407	**WP**	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [μm]		D95 [μm]		D95 [μm]	
Day0	3.30	3.83	3.34	3.83	3.30	3.83
Day1	6.00	6.30	6.20	6.94	9.10	11.80
Day7	8.00	10.90	10.31	18.08	12.20	25.70
Day14	14.60	17.90	22.06	19.10	11.70	21.80
Month	16.00	15.30	20.25	35.40	10.30	20.20

Stabilization of nanosuspension with combination of vitamin E TPGS with HPMC caused slight fluctuation of particle size around D95 value of 1.5 μm and no larger particles or aggregates were detected (Table X). Light microscopy confirmed these results.

Table X: Results of LD analysis of dexamethasone nanosuspensions, stabilized with vitamin E TPGS and HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS	4°C		25°C		40°C	
+ HPMC	**WP**	**P**	**WP**	**P**	**WP**	**P**
LD	D95 [μm]		D95 [μm]		D95 [μm]	
Day0	1.50	1.11	1.50	1.11	1.50	1.11
Day1	1.38	1.47	1.15	1.15	1.04	2.55
Day7	0.96	0.94	1.73	1.06	1.05	1.08
Day14	1.02	0.99	1.56	1.48	1.23	1.15
Month	1.21	1.57	1.46	1.43	1.42	1.41

4.2.3 Zeta potential

Nanosuspensions stabilized with poloxamer 188 exhibited ZP around -30 mV on day 0 and day 1 (Table XI), which is quite high for particles stabilized with an adsorbed non-ionic stabilizer. The data indicate that the stabilizer layer was relatively thin and provided poor stabilization (8), what was clearly presented in the short-term stability test. The ZP value remained between -27.0 and -22.0 mV after 1 month, therefore the formulation could have been physically stable. However, increased average particle size and high value of PI indicate the formulation was not physically stable (Figure 10).

Table XI: Average zeta potential of dexamethasone nanosuspensions, stabilized with poloxamer 188 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

P188	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day 0	-30.6	-33.9	-30.6	-33.9	-30.6	-33.9
Day 1	-30.3	-36.1	-30.1	-29.8	-32.1	-30.2
Day 7	-18.8	-18.1	-21.8	-21.2	-21.1	-20.7
Day 14	-17.5	-21.1	-15.4	-19.3	-19.3	-22.6
Month	-25.3	-22.7	-24.5	-23.8	-27.4	-25.2

In contrast to nanosuspensions stabilized with poloxamer 188, nanosuspensions stabilized with poloxamer 407 demonstrated less negative ZP values (around -20 mV) on day 0 (Table XII). Similar values were found in the literature and they were shown to be sufficient to obtain a stable product (8). ZP values remained more or less the same in short-term stability test. In conclusion, the dispersed particles were physically stable within the timespan of one month.

Table XII: Average zeta potential of dexamethasone nanosuspensions, stabilized with poloxamer 407 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

P407	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-21.4	-20.9	-21.4	-20.9	-21.4	-20.9
Day1	-21.6	-20.3	-22.4	-22.1	-21.2	-22.6
Day7	-17.4	-18.6	-18.7	-20.0	-23.2	-23.9
Day14	-22.6	-22.5	-22.6	-21.4	-24.3	-23.0
Month	-23.7	-23.0	-21.0	-22.0	-25.8	-24.9

ZP were low for nanosuspensions stabilized with HPMC throughout investigated time period (Table XIII), what is typical for sterically stabilized particles (1). Nevertheless, when stabilization is based on steric stabilizers, the ZP is not relevant parameter for the evaluation of long-term stability of formulation (3). In case of HPMC stabilized nanosuspensions absolute values of ZP of 20 mV or lower can provide sufficient stabilization due to the steric effect (8).

Table XIII: Average zeta potential of dexamethasone nanosuspensions, stabilized with 0.8% HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

HPMC	4°C		25°C		40°C	
0.8%	**WP**	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-8.9	-9.4	-8.9	-9.4	-8.9	-9.4
Day1	-5.0	-5.5	-5.8	-6.4	-4.8	-6.3
Day7	-3.7	-3.5	-4.8	-4.6	-3.8	-2.6
Day14	-8.0	-11.0	-12.3	-9.2	-10.3	-9.3
Month	-6.3	-7.3	-5.1	-4.2	-3.5	-4.4

Nanosuspensions stabilized with 0.1% HPMC exhibited comparable ZP as nanosuspensions stabilized with 0.8% HPMC. ZP values decreased during the short-term stability test (Table XIV). Nanosuspensions were physically stable throughout one month according to the PCS and LD results (Figure 13, Table VI).

Table XIV: Average zeta potential of dexamethasone nanosuspensions stabilized with 0.1% HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

HPMC		
0.1%	**WP**	**P**
	ZP [mV]	
Day0	-10.5	-9.05
Day1	-13.3	-11.2
Day7	-15.85	-9.11
Day14	-10.55	-8.52
Month	-3.5	-3.94

In case of nanosuspensions stabilized with vitamin E TPGS absolute values of ZP were slightly higher than 20 mV after the milling process, which provides only short-term physical stability (8). The absolute ZP values increased over the time (Table XV).

Table XV: Average zeta potential of dexamethasone nanosuspensions, stabilized with vitamin E TPGS over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-20.4	-21.1	-20.4	-21.1	-20.4	-21.1
Day1	-23.9	-24.8	-26.2	-24.9	-23.9	-24.9
Day7	-21.3	-21.3	-21.8	-23.8	-21.4	-23.8
Day14	-27.0	-21.1	-24.6	-24.7	-24.5	-24.7
Month	-23.8	-22.9	-25.3	-25.8	-25.6	-25.8

Nanosuspensions stabilized with the combination of vitamin E TPGS and poloxamer 188 exhibited after the milling similar ZP as nanosuspensions stabilized with vitamin E TPGS alone. Similar to vitamin E TPGS stabilized nanosuspension absolute values of ZP of nanosuspensions stabilized with the combination of stabilizers increased over the time, however, the increase in ZP values was larger (Table XVI).

Table XVI: Average zeta potential of dexamethasone nanosuspensions stabilized with vitamin E TPGS and poloxamer 188 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS + P188	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-20.7	-24.3	-20.7	-24.3	-20.7	-24.3
Day1	-25.0	-25.1	-25.5	-24.3	-8.2	-5.1
Day7	-26.1	-24.9	-30.5	-27.0	-30.7	-27.5
Day14	-26.3	-27.1	-26.8	-28.6	-28.2	-28.5
Month	-28.6	-28.6	-29.5	-29.1	-33.1	-28.7

Right after production, ZP of nanosuspension stabilized with combination of vitamin E TPGS and poloxamer 407 was around -24 mV (Table XVII), which presented only short-term physical stability. However, the absolute values of ZP did not increase over the time in this case, but they seem to be relatively stable.

Table XVII: Average zeta potential of dexamethasone nanosuspensions stabilized with vitamin E TPGS and poloxamer 407 over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS + P407	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-24.1	-23.8	-24.1	-23.8	-24.1	-23.8
Day1	-15.0	-21.5	-13.4	-17.9	-18.3	-21.7
Day7	-25.0	-21.8	-18.5	-21.8	-21.7	-20.0
Day14	-23.9	-23.8	-24.8	-24.4	-24.4	-24.4
Month	-26.1	-24.5	-20.5	-25.4	-29.6	-26.4

Due to the HPMC properties absolute ZP values of nanosuspensions stabilized with combination of vitamin E TPGS and HPMC were significantly lower compared to vitamin E TPGS stabilized nanosuspensions and lower than ZP of particles stabilized only with HPMC (Tables XIII, XV, XVIII). The combination of stabilizers could still assure long-term physical stability of formulation due to the steric effect of HPMC.

Table XVIII: Average zeta potential of dexamethasone nanosuspensions stabilized with vitamin E TPGS and HPMC over time period of one month (30 days). WP – nanosuspensions without added preservative; P – nanosuspensions with added preservative.

TPGS +	4°C		25°C		40°C	
	WP	**P**	**WP**	**P**	**WP**	**P**
	ZP [mV]		ZP [mV]		ZP [mV]	
Day0	-6.2	-5.8	-6.2	-5.8	-6.2	-5.8
Day1	-7.7	-7.7	-5.3	-5.7	-8.1	-8.3
Day7	-3.1	-4.5	-3.5	-5.2	-5.0	-4.1
Day14	-3.4	-3.6	-3.1	-3.3	-4.5	-6.3
Month	-4.1	-4.2	-5.1	-5.5	-4.5	-2.4

4.2.4 Light microscopy

In this section only the most representative images of investigated nanosuspensions are shown (Figure 18), due to too many photos taken during the research work.

Figure 18A was taken at 400-fold magnification without polarized light. The nanosuspension shown has a homogenous appearance. Neither large particles nor aggregates can be observed.

The nanosuspension shown in Figure 18B was taken at 600-fold magnification without polarized light. It looks slightly inhomogeneous, since a few loose aggregates can be seen. LD data (Table III) show a relatively low D95 value indicating that aggregates were broken down with stirrer used in laser diffractometer.

Figure 18C was taken at 400-fold magnification with polarized light. Nanocrystals present in the sample appear as white dots on dark background, giving an impression of a ”starry sky”, confirming the presence of nanocrystals in the sample.

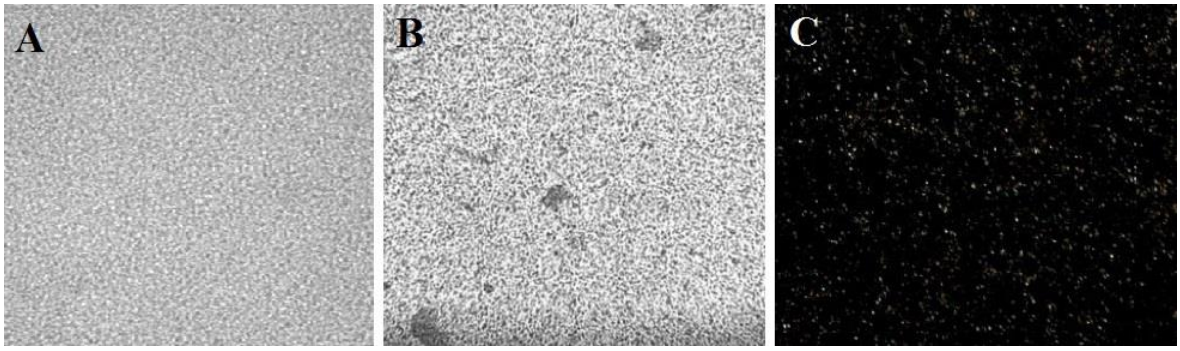

Figure 18: (A) 0.8% (w/w) HPMC stabilized nanosuspension on day 1 after production, (B) 0.8% (w/w) poloxamer 188 stabilized nanosuspension on day 14 after production, (C) 0.8% (w/w) poloxamer 188 stabilized nanosuspension on day 1 after production. All three samples were stored at 25°C.

According to the results of the short-term physical stability test the investigated stabilizers and their combinations can be classified in four groups.

To the first group belong both tested concentrations of HPMC and the combination of vitamin E TPGS and HPMC. These stabilizers exhibited similar trends in physical stability test, since particle size did not show any or only minimal growth with time, the particle size distribution was stable throughout the investigated time period or it even decreased with time. LD analysis showed no big increase in particle size either nor presence of any particle aggregates. ZPs were typically low due to the properties of HPMC.

The second group represents poloxamer 407. The nanosuspensions stabilized with this stabilizer did not exhibit any significant increase in average particle size or PI throughout the tested time period. Furthermore, the particle size in some samples even decreased with time. The average particle size in these samples was the smallest and most homogenous after the short-term stability test. Absolute values of ZPs were high enough, according to the

literature, to provide physical stability of formulations and they seem to increase with time. According to the ZP values and particle size distribution these formulations exhibit good physical stability. However, according to the LD and light microscopy results, some aggregates were found in these samples after one month of storage. As reported in literature, a slight increase in particle size of nanosuspensions is not considered to impair their dermal application.

To the third group of stabilizers belong vitamin E TPGS and its combinations with poloxamer 188 and 407. Nanosuspensions stabilized with these stabilizers have all shown similar tendencies. Their particles grew with increased temperature, confirmed also by LD analysis. Nanosuspensions exhibited similar initial absolute ZP values, which were stable or even increased during the short-term stability test.

And the last stabilizer, poloxamer 188 belongs to the fourth group. Nanosuspensions stabilized with this stabilizer demonstrated a significant increase in average particle size and size distribution in the physical stability test. Furthermore, aggregation was observed at 25°C and 40°C. ZPs of nanosuspensions indicate their physical stability during the short-term stability test, however, PI was just too high to ensure long-term stability.

The aggregated particles are not desired in dermal products, since they do not show improved properties of nanosized particles. However, nanosuspensions containing some particle aggregates can still be usable in dermal products in case the aggregation does not continue. After addition of the nanocrystals to dermal formulation, the nanocrystals are additionally stabilized by the high viscosity of the formulation (8). Anyway, the commercial formulation needs to exhibit long-term stability for at least two years (8), which can be tested in advance by accelerated stability studies.

4.3 Selection of preservative and its influence on nanosuspension particle size

Successful preservation of nanosuspensions depends on various factors e.g., properties of particle surface, stabilizer hydrophobicity and preservative hydrophilicity. The effect of preservative on nanosuspension stability should be evaluated since preservative can impair the nanosuspension stability and as a consequence the particles tend to grow. After consideration propylene glycol, benzyl alcohol and a combination of sorbic acid and potassium sorbate were chosen as preservatives in our nanosuspension formulations. In the

initial screening only 10% propylene glycol was used as a preservative. Afterwards other concentrations of propylene glycol and other preservatives were tested as well.

4.3.1 Influence of preservative on particle size and particle size distribution

Nanosuspensions stabilized with poloxamer 188 or HPMC exerted no difference in particle size between preserved (with propylene glycol) and non-preserved samples (Figures 10, 12 and 13, Tables III, V, VI, XI, XIII and XIV). Nanosuspension stabilized with poloxamer 407 and preserved with propylene glycol showed similar results (size and PI) (Figure 11), however, LD analysis revealed a slightly bigger value of D95 for preserved compared to non-preserved sample (Table IV). Nanosuspensions stabilized with vitamin E TPGS or its combinations with poloxamers indicated a prominent increase in particle size in case of preservative added (Figures 14, 15, 16). The only exception was the nanosuspension stabilized with combination of vitamin E TPGS and HPMC, which exhibited a smaller increase in particle size compared to non-preserved samples (Figure 17).

Poloxamer 407 was the stabilizer of choice to be tested in combination with propylene glycol as preservative in greater details and also combined with other preservatives. The particle size in nanosuspensions increased with time and with higher percentage of propylene glycol added (Figure 19). On day seven after nanosuspension preparation and onwards there was a prominent increase in particle size in case of nanosuspensions with 18% (w/w) and 20% (w/w) propylene glycol added. Particle size distribution increased as well. According to the obtained data it can be concluded that nanosuspensions preserved with propylene glycol in concentration up to 16% (w/w) are short-term physically stable.

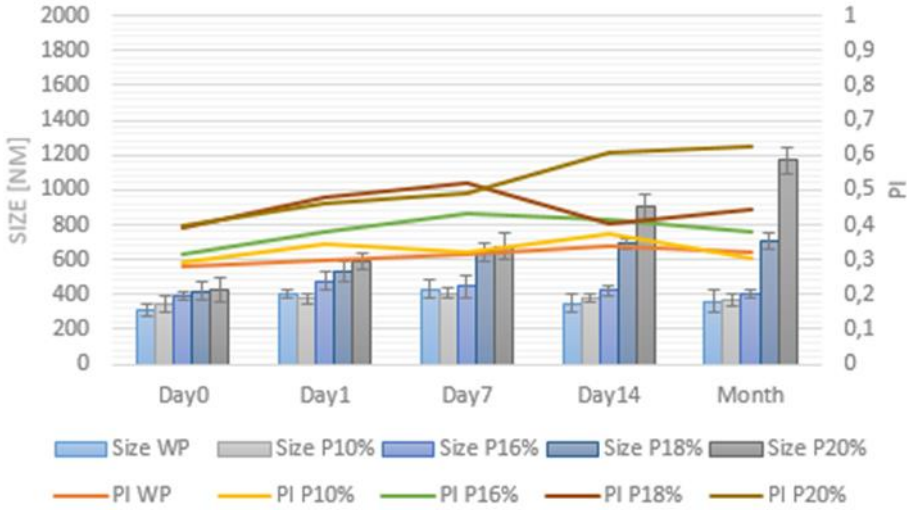

Figure 19: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with poloxamer 407 over time period of 30 days. WP – without preservative, P10-20% - amount of propylene glycol added.

Nanosuspension stabilized with poloxamer 407 and preserved with combination of sorbic acid and potassium sorbate showed stable particle size and particle size distribution until day 7 after preparation, later both increased steadily. Nanosuspension preserved with benzyl alcohol showed small increase in particle size and particle size distribution up to day 7 after preparation, afterwards significant particle growth was observed (Figure 20). Particle growth was due to the preservative added, which hinders the steric stabilizer layer, therefore its presence reduces the hydrophobic interactions of stabilizer with particles, resulting in particle growth. This disadvantage can be used in other fields as an advantage e.g., benzyl alcohol can be used as an enhancer of fluidity of surfactant layer in liposomes (42).

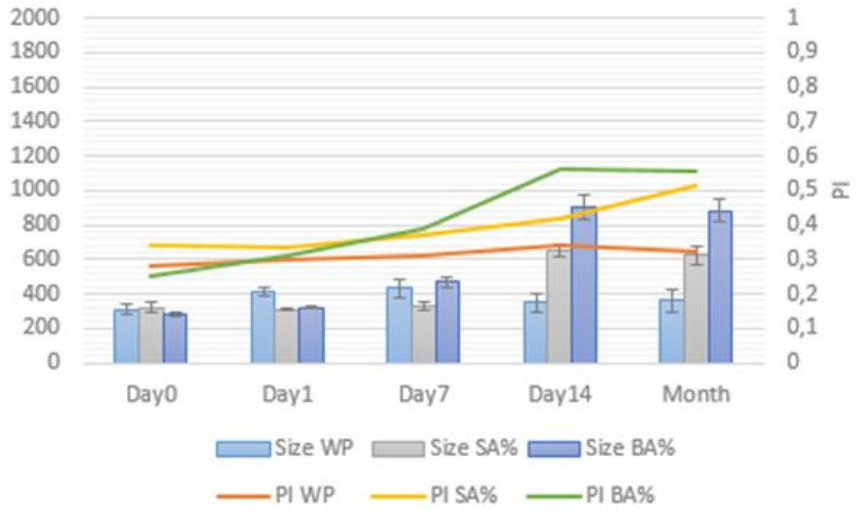

Figure 20: Average particle size and polydispersity index (PI) of dexamethasone nanosuspensions, stabilized with poloxamer 407 over time period of one month (30 days). WP – without preservative, SA – combination of sorbic acid and potassium sorbate, BA – benzyl alcohol.

4.3.2 Influence of preservative on zeta potential

Nanosuspensions stabilized either with vitamin E TPGS alone or in combination with other stabilizers did not show any differences in ZPs between preserved and non-preserved samples (Tables XV, XVI, XVII, XVIII). ZP values did not change significantly when different percentage of propylene glycol was added (Table XIX). Nanosuspension preserved with combination of sorbic acid and potassium sorbate exhibited a lower absolute initial value of ZP (16 mV), which further decreased during the short-term stability test (8.2 mV) (Table XIX). Absolute value of ZP of benzyl alcohol preserved nanosuspension was high after the production (30.2 mV), which could provide physical stability of formulation. However, it decreased slightly until the end of the short-term stability test (24.6 mV).

Table XIX: Zeta potential of dexamethasone nanocrystals, stabilized with poloxamer 407 over time period of one month (30 days). WP – nanosuspension without added preservative; P10-20% - amount of propylene glycol added, SA –sorbic acid/potassium sorbate, BA – benzyl alcohol.

ZPs	Day 0	Day 1	Day 7	Day 14	Day 30
WP	-23.3	-22	-17.8	-22.7	-23.4
PG10%	-22.9	-25.6	-22.1	-23.43	-23.0
PG16%	-24.3	-25.9	-20.0	-22.8	-22.0
PG18%	-23.1	-22.2	-20.1	-23.1	-21.3
PG20%	-25.1	-25.2	-22.6	-27	-20.5
BA	-30.2	-28.3	-25.7	-29.5	-24.6
SA	-16.3	-10.8	-8.9	-9.3	-8.2

These results demonstrate that added preservatives affect the thickness of the stabilizing layer on particle surface and its stabilizing ability. Nanosuspensions stabilized with poloxamer 188, poloxamer 407 and HPMC were the least affected by the initially tested stabilizer, namely propylene glycol. However, the most physically stable dispersion with added propylene glycol was sample stabilized with poloxamer 407. Propylene glycol was shown to promote particle growth when vitamin E TPGS was used as a stabilizer, alone or in a combination with other stabilizers. The smallest effect on the physical stability of nanosuspensions during short-term stability test showed among all investigated preservatives propylene glycol in the concentration range 10-16% (w/w). Combination of sorbic acid and potassium sorbate as well as benzyl alcohol exert higher lipophilicity compared to propylene glycol and thus had a major impact on the stability of nanosuspensions. Besides the observed destabilization of nanosuspensions, adsorption of preservatives to the particle surface in large amount can result in reduced preservative efficacy, which was tested in microbial assay.

4.4 Preservative efficacy

The drugs for dermal administration should have a low bacterial count. According to the European Pharmacopoeia the acceptance criteria for non-sterile pharmaceutical products is based upon the total aerobic microbial count (TAMC), which is 10^2 per gram of formulation, and the total combined yeasts/moulds count (TYMC), which is 10^1 per gram of formulation. Absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* is required as well (43). Higher risk of microbial contamination is typically associated with water containing

products. The time span the product is suitable for use after opening should be defined for such products (37).

Table XX: Nanosuspensions in microbial stability test, results are in days when the growth of colonies was observed. C- - negative control, C+ - positive control, PR – probe, S – small colony, L – large colony, I – intensive microbial growth, number in brackets represent an approximate number of colonies present, the same shade of grey demonstrates the same result as the day before.

NS:	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
P 188: C-							
C+	S (<20)				I		
PR			S (1)	S (<20)	I		
P 407: C-							
C+	S (50+)	I					
PR		S(<50)					I
HPMC: C-							
C+	S (<20)				I		
PR			S (<20)		Less I	I	
TPGS: C-							
C+		S (<50)				L (50+)	
PR		S (<20)				4 L + S	

Initial microbial testing revealed that propylene glycol added was not effective. According to the literature data, the preservative generally recommended for preservation of formulations with different drugs and stabilizers is 10% (w/w) of propylene glycol. In our case, the stabilizer most likely prevented the effect of preservative added. Due to the intensive colony growth the agar plates were thrown away before the end of the assay (14 days of storage in the warm and dry oven). Agar plates were streaked with nanosuspension without preservative (positive control), nanosuspension with preservative (probe) and pure preservative (negative control) (Figure 21, Table XX).

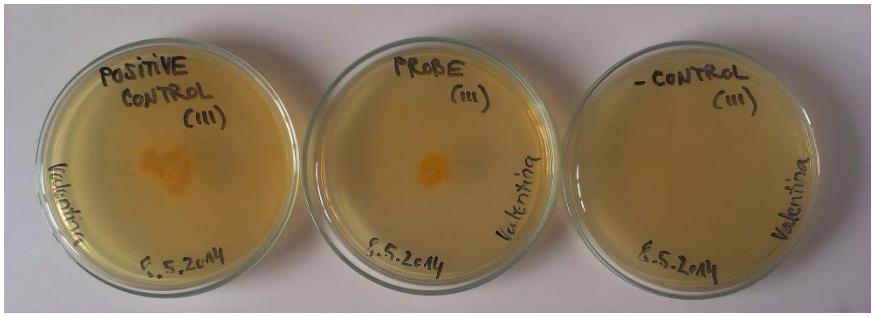

Figure 21: Agar plates five days after streaking with nanosuspensions stabilized with HPMC: nanosuspension without preservative (positive control), nanosuspension with preservative (probe) and pure preservative (negative control).

Agar plates streaked with nanosuspensions stabilized with vitamin E TPGS or its combinations remained clear and colony-free for a slightly longer time period compared to other samples. The best result was obtained in case of nanosuspension stabilized with combination of Vitamin E TPGS and poloxamer 407 (Tables XX and XXI).

*Table XXI: Nanosuspensions in microbial stability test, results are in days when the growth of colonies was observed. C- - negative control, C+ - positive control, PR – probe, S – small colony, L – large colony, I – intensive growth, number in brackets represent an approximate number of colonies present, same shade of grey demonstrates the same result as the day before, *12th day - change of result on the 12th day.*

TPGS +	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
P188: C-	/	/	/	/	/	/	/
C+	S(<30)			I			
PR	/	/	3 S		3 L + S		
P407: C-	/	/	/	/	/	/	/
C+	/	/	/	4 S			
PR	/	/	/	/	/	/	*12th day: S
HPMC:C-	/	/	/	/	/	/	/
C+	/	S (<20)					
PR	/	/	/	/	/	/	3 S

Nanosuspensions stabilized with 0.1% (w/w) HPMC were tested with addition of different percentage of propylene glycol as preservative (Table XXII). The sample preserved with 10% propylene glycol and positive control exhibited some microbial growth on the fifth day after streaking, while the samples preserved with 18% and 20% of propylene glycol did not show any microbial growth throughout 14 days.

Table XXII: Nanosuspensions in microbial stability test, results are in days when the growth of colonies was observed. C- - negative control, C+ - positive control, PR – probe, S – small colony, L – large colony, I – intensive growth, number in brackets represent an approximate number of colonies present, same shade of grey demonstrates the same result as the day before.

HPMC	**Day 1**	**Day 2**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 14**
C-	/	/	/	/	/	/	/
C+	/	/	/	L + S			
PG10%	/	/	/	15 S			
PG18%	/	/	/	/	/	/	/
PG20%	/	/	/	/	/	/	/

In case of nanosuspension stabilized with the stabilizer of choice, namely poloxamer 407, it was demonstrated that percentage of propylene glycol in formulation needs to be at least 16% (w/w) to inhibit any microbial growth. Lower percentages (10-14% (w/w)) revealed some microbial growth after three days of microbial testing (Table XXIII).

Table XXIII: Nanosuspensions in microbial stability test, results are in days when the growth of colonies was observed. C- - negative control, C+ - positive control, PR – probe, S – small colony, L – large colony, I – intensive growth, number in brackets represent an approximate number of colonies present, same shade of grey demonstrates the same result as the day before.

P 407	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 9**	**Day 14**
C-	/	/	/	/	/		
PG10%	/	/	4 S	4 L		I	
PG14%	/	/	S (<50)				I
PG16%	/	/	/	/	/	/	/
PG18%	/	/	/	/	/	/	/
PG20%	/	/	/	/	/	/	/
SA	/	/	/	/	/	S	/
BA	/	/	/	/	/	S	/

According to the obtained results it can be concluded that 16% (w/w) of propylene glycol was sufficient to preserve microbial stability of nanosuspensions stabilized with poloxamer 407. Agar plates streaked with nanosuspensions stabilized with poloxamer 407 and preserved with sorbic acid/potassium sorbate or benzyl alcohol did not exhibit any microbial growth in the time frame of at least eight days.

4.5 Amount of dexamethasone dissolved in the dispersion medium of nanosuspensions

Firstly, the calibration curve was prepared based on the drug stock solution to calculate the amount of dexamethasone in the water phases. Different dilutions of drug stock solution were prepared and their absorbance was measured spectrophotometrically. The results are the average of two measurements of every dilution (Figure 22).

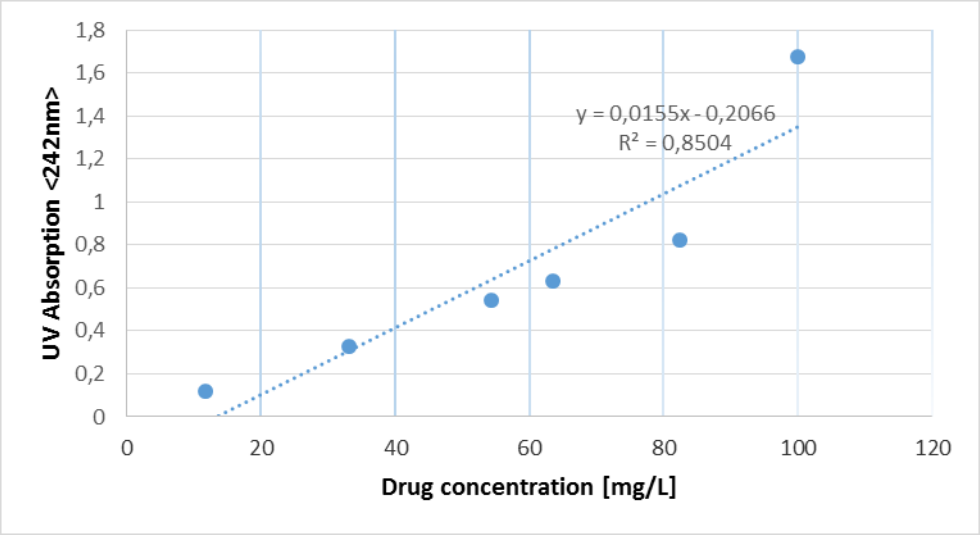

Figure 22: Calibration curve of dexamethasone in distilled water.

Absorption spectrum of 0.8% (w/w) aqueous solution of stabilizer was measured to exclude presence of any stabilizer peaks at analytical wavelength. The collected data (Table XXIV) show that the smallest amount of drug was dissolved in dispersion medium of nanosuspensions stabilized with HPMC, followed by nanosuspensions stabilized with poloxamer 407 and the highest amount was dissolved in dispersion medium of nanosuspensions stabilized with poloxamer 188. Samples stored at 40°C had more drug dissolved in the aqueous medium compared to nanosuspensions stored at lower temperatures. Nanosuspensions with added propylene glycol had more dissolved dexamethasone in dispersion medium than nanosuspensions without it (Table XXIV). However, there were some exceptions, namely nanosuspension stabilized with poloxamer 188, stored at 40°C and nanosuspension stabilized with poloxamer 407, stored at 25°C. Unfortunately, some samples were not possible to measure, due to the lack of sample quantity or due to the stabilizer chemical properties as already described in the section *Materials and methods.*

Table XXIV: Concentration of dexamethasone dissolved in dispersion medium of nanosuspensions stabilized with poloxamer 188 (10 weeks after preparation), poloxamer 407 and HPMC (9 weeks after preparation). WP – sample without preservative, P – sample with preservative.

		Concentration of dissolved drug [mg/L]
Poloxamer 188	WP 25°C	687.2
	P 25°C	740.9
	WP 4°C	606.0
	WP 40°C	993.4
	P 40°C	633.0
Poloxamer 407	WP 25°C	674.3
	P 25°C	544.0
	WP 4°C	816.5
	P 4°C	832.3
	P 40°C	1050.6
HPMC	WP 4°C	374.2
	P 4°C	519.0
	P40°C	519.2

On the other hand, the data which were obtained right after production of nanosuspension, demonstrated higher amounts of dissolved drug in the aqueous medium (Table XXV). Most probably that was due to oversaturation of dispersion medium with drug after preparation of nanosuspensions or the effect of particle size on drug solubility.

Table XXV: UV measurements of repeated batch of Poloxamer 407, two weeks after production. WP – sample without preservative, PG – sample with propylene glycol, SA - sample with a combination of sorbic acid and potassium sorbate, BA - sample with benzyl alcohol, numbers indicate amount (w/w) of the preservative in the nanosuspension.

		Concentration of dissolved drug [mg/L]
Poloxamer 407	WP20% 25°C	1204.5
	PG20% 25°C	1540.8
	SA 25°C	2034.8
	BA0.5% 25°C	2012.1
	WP18% 4°C	1192.1
	PG18% 4°C	1389.0
	SA 4°C	2027.9
	WP10% 40°C	1318.3
	PG10% 40°C	1563.0
	SA 40°C	2013.8

5 CONCLUSIONS

Dexamethasone nanosuspensions were produced with pearl milling with the purpose to achieve a higher solubility and dissolution velocity of the drug. Four stabilizers, appropriate for dermal application, were studied as well as the influence of different preservatives, storage conditions and time on nanosuspension properties.

The most effective particle size reduction was observed in nanosuspensions stabilized with a combination of vitamin E TPGS and poloxamer 407 (average particle size 334 nm, PI around 0.3). After short-term stability test stabilizers were classified in four groups according to their ability to maintain physical stability of nanosuspensions. To the first group belong HMPC and its combination with vitamin E TPGS, which assured the best physical stability of nanosuspensions. These nanosuspensions could exhibit long-term physical stability despite the absolute zeta potential was smaller than 30 mV, due to steric properties of HPMC. The only drawback of this formulation was relatively big average particle size (around 600 nm) after pearl milling. To the second group belongs poloxamer 407, which enabled preparation of nanosuspension with the smallest average particle size after the short-term stability test and with sufficient ZP to provide good short-term stability. To the third group of stabilizers belong vitamin E TPGS and its combinations with poloxamer 188 and 407. Finally, to the fourth group belongs poloxamer 188, since its application resulted in a significant increase in average particle size and size distribution in short-term stability test.

It was important to evaluate the influence of preservative on nanosuspension stability, since preservative can impair the nanosuspension stability and consequently causes particle growth. Nanosuspension stabilized with poloxamer 407 was the most physically stable dispersion with added propylene glycol. Among the investigated preservatives propylene glycol was shown to have the smallest effect on physical stability of nanosuspensions, therefore, it was the preservative of choice in a concentration range of 10 to 16% (w/w). Besides the effect of preservative on nanosuspension stability, its adsorption to the particle surface can result in reduced antimicrobial effect, which was tested in microbial test. It revealed that nanosuspension stabilized with poloxamer 407 require at least 16% (w/w) of propylene glycol to prevent bacteria growth in time frame of fourteen days. Nanosuspensions preserved with a combination of sorbic acid and potassium sorbate or benzyl alcohol did not exhibit any bacterial growth in the time frame of at least eight days.

UV measurements revealed that the smallest amount of dissolved drug was present in nanosuspensions stabilized with HPMC, followed by nanosuspensions stabilized with poloxamer 407 and poloxamer 188.

To sum up, the results of our study have shown that the most promising dexamethasone nanosuspension formulation is the one stabilized with 0.8% (w/w) of poloxamer 407 and preserved with 16% (w/w) of propylene glycol.

6 REFERENCES

1. Ghosh I, Bose S, Vippagunta R, Harmon F: Nanosuspension for improving the bioavailability of a poorly soluble drug and screening of stabilizing agents to inhibit crystal growth. International journal of pharmaceutics, 2011, 409.1: 260-268.
2. Rabinow, Barrett E: Nanosuspensions in drug delivery. Nature Reviews Drug Discovery, 2004, 3.9: 785-796.
3. Peltonen L, Hirvonen J: Pharmaceutical nanocrystals by nanomilling: Critical process parameters, particle fracturing and stabilization methods. J Pharm Pharmacol 2010; 62(11): 1569-79.
4. Filippos Kesisoglou, Santipharp Panmai, Yunhui Wu: Nanosizing — Oral formulation development and biopharmaceutical evaluation, Advanced Drug Delivery Reviews, Vol 59, Issue 7, 2007, Pages 631-644
5. Merisko-Liversidge Elaine M, Liversidge G: Drug nanoparticles: formulating poorly water-soluble compounds. Toxicologic pathology, 2008, 36.1: 43-48.
6. Junghanns JUA, Müller RH: Nanocrystal technology, drug delivery and clinical applications. International journal of nanomedicine, 2008, 3.3: 295.
7. Müller, Rainer H; Keck, Cornelia M: Challenges and solutions for the delivery of biotech drugs–a review of drug nanocrystal technology and lipid nanoparticles. Journal of biotechnology, 2004, 113.1: 151-170.
8. Mishra PR, Shaal LA, Müller RH, & Keck CM: Production and characterization of Hesperetin nanosuspensions for dermal delivery. International journal of pharmaceutics, 2009, 371.1: 182-189.
9. Pardeike J: Nanosuspensions and nanostructured lipid carriers for dermal application. 2009. PhD Thesis. Freie Universität Berlin, Germany.
10. Zhai X, Lademann, J, Keck CM, Müller RH: Dermal nanocrystals from medium soluble actives–Physical stability and stability affecting parameters. European Journal of Pharmaceutics and Biopharmaceutics, 2014, 88.1: 85-91.
11. Müller RH, Gohla S, Keck CM: State of the art of nanocrystals–special features, production, nanotoxicology aspects and intracellular delivery. European Journal of Pharmaceutics and Biopharmaceutics, 2011, 78.1: 1-9.

12. Shaal LA, Müller RH, Keck CM: Preserving hesperetin nanosuspensions for dermal application. Die Pharmazie-An International Journal of Pharmaceutical Sciences, 2010, 65.2: 86-92.
13. Gao L, Zhang D, Chen M: Drug nanocrystals for the formulation of poorly soluble drugs and its application as a potential drug delivery system. Journal of Nanoparticle Research, 2008, 10.5: 845-862.
14. Keck CM, Müller RH: Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation. European Journal of Pharmaceutics and Biopharmaceutics, 2006, 62.1: 3-16.
15. Salazar J, Ghanem A, Müller RH, Möschwitzer JP: Nanocrystals: comparison of the size reduction effectiveness of a novel combinative method with conventional top-down approaches. European Journal of Pharmaceutics and Biopharmaceutics, 2012, 81.1: 82-90.
16. Scholz P, Arntjen A, Müller RH, Keck CM: ARTcrystal® process for industrial nanocrystal production—Optimization of the ART MICCRA pre-milling step. International journal of pharmaceutics, 2014, 465.1: 388-395.
17. Chin WWL, Parmentier J, Widzinski M, Tan EH, Gokhale R: A brief literature and patent review of nanosuspensions to a final drug product. Journal of pharmaceutical sciences, 2014, 103.10: 2980-2999.
18. Sharma R, Jain S, Tiwari R: Nanoparticle technology: formulating poorly water-soluble compounds. International Journal of Pharmaceutical Sciences and Research, 2015, 6.1: 57.
19. Shegokar R, Müller RH: Nanocrystals: industrially feasible multifunctional formulation technology for poorly soluble actives. International journal of pharmaceutics, 2010, 399.1: 129-139.
20. Salazar J, Müller RH, Möschwitzer JP: Combinative particle size reduction technologies for the production of drug nanocrystals. Journal of Pharmaceutics, 2014.
21. Liang Y, Binner J: Effect of triblock copolymer non-ionic surfactants on the rheology of 3mol% yttria stabilised zirconia nanosuspensions. Ceramics international, 2008, 34.2: 293-297.
22. Vernon-Parry KD: Scanning electron microscopy: an introduction. III-Vs Review, 2000, 13.4: 40-44.

23. Vernon-Perry KD: TEM: an introduction. III-Vs Review, 2000, 13.6: 36-40.
24. http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Dexamethasone_Alapis_articlce29/WC500121862.pdf (Accessed on Aug 20th 2015)
25. Spangler M, Mularz E: A validated, stability-indicating method for the assay of dexamethasone in drug substance and drug product analyses, and the assay of preservatives in drug product. Chromatographia, 2001, 54.5-6: 329-334.
26. http://www.gd-online.de/german/veranstalt/images2010/GD_Wirkstoffdossiers_Version_19.11.2009.pdf (Accessed on May 30th 2014)
27. Dexamethasone (PubChem CID 5743) http://pubchem.ncbi.nlm.nih.gov/compound/5743#section=Color (Accessed on Aug 20th 2014)
28. Kassem MA, Rahman AA, Ghorab MM, Ahmed MB, Khalil RM: Nanosuspension as an ophthalmic delivery system for certain glucocorticoid drugs. International journal of pharmaceutics, 2007, 340.1: 126-133.
29. Baba K, Nishida K, Hashida N: Method for producing an aqueous dispersion of drug nanoparticles and use thereof. U.S. Patent Application 14/395,952, 2013.
30. Baba K, Nishida K: Steroid nanocrystals prepared using the Nano Spray Dryer B-90. Pharmaceutics, 2013, 5.1: 107-114.
31. Gan L et al.: Self-assembled liquid crystalline nanoparticles as a novel ophthalmic delivery system for dexamethasone: improving preocular retention and ocular bioavailability. International journal of pharmaceutics, 2010, 396.1: 179-187.
32. Van Eerdenbrugh B, Van den Mooter G, Augustijns P: Top-down production of drug nanocrystals: nanosuspension stabilization, miniaturization and transformation into solid products. International journal of pharmaceutics, 2008, 364.1: 64-75.
33. http://www.pharma-ingredients.basf.com/Statements/Technical%20Informations/EN/Pharma%20Solutions/03_100102e_Lutrol%20L%20and%20Lutrol%20F-Grades.pdf (Accessed on Aug 20th 2014)
34. http://www.sigmaaldrich.com/catalog/product/sigma/56340?lang=en&region=SI (Accessed on Aug 20th 2014)
35. Mu L, Feng SS: Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for

controlled release of paclitaxel (Taxol®). Journal of Controlled Release, 2002, 80.1: 129-144.

36. http://www.sigmaaldrich.com/catalog/product/sigma/57668?lang=en&region=SI (Accessed on Aug 20th 2014)
37. Daniels R, Knie U: Galenics of dermal products–vehicles, properties and drug release. JDDG: Journal der Deutschen Dermatologischen Gesellschaft, 2007, 5.5: 367-383.
38. Obeidat WM, Schwabe K, Müller RH, Keck CM: Preservation of nanostructured lipid carriers (NLC). European Journal of Pharmaceutics and Biopharmaceutics, 2010, 76.1: 56-67.
39. Kobierski S, Ofori-Kwakye K, Müller RH, Keck CM: Resveratrol nanosuspensions: interaction of preservatives with nanocrystal production. Die Pharmazie-An International Journal of Pharmaceutical Sciences, 2011, 66.12: 942-947.
40. http://www.malvern.com/en/products/product-range/zetasizer-range/zetasizer-nano-range/zetasizer-nano-z/default.aspx?gclid=CjwKEAjwgqWvBRC2kajZjqeOn0oSJADqBi-lmSVuCeSVosQUC0nVRvS7zxwpAZcV28NlTuRZsjrWvhoCrR_w_wcB (Accessed on Sep 4th 2015)
41. Dai WG, Dong LC, Li S, Deng Z: Combination of Pluronic/Vitamin E TPGS as a potential inhibitor of drug precipitation. International journal of pharmaceutics, 2008, 355.1: 31-37.
42. Chen Y et al.: Enhanced bioavailability of the poorly water-soluble drug fenofibrate by using liposomes containing a bile salt. International journal of pharmaceutics, 2009, 376.1: 153-160.
43. Council of Europe. European Pharmacopoeia. 8th ed. Strasbourg: Council of Europe; 2007:185-189.