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Development of an Itraconazole-loaded nanostructured lipid carrier (NLC) formulation for pulmonary application

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ABSTRACT

Itraconazole-loaded NLC for pulmonary application were developed. In Precirol ATO 5 and oleic acid Itraconazole had the highest solubility. The solid lipid and the oil were mixable in a ratio 9:1 possessing a melting point above body temperature. 0.4% Itraconazole was dissolved in this lipid blend. Eumulgin SLM 20 was the stabilizer with the highest affinity to the lipid blend used as particle matrix. 2.5% Eumulgin SLM 20 was sufficient to obtain NLC with a narrow particle size distribution and sufficient stability. The tonicity of the formulation was adjusted with glycerol. Sterility was obtained by autoclaving. Neither the addition of glycerol nor autoclaving had an influence on the particle size and the zeta potential of Itraconazole-loaded NLC. SEM images showed spherical particles confirming the particle size measured by light scattering techniques. An entrapment efficiency of 98.78% was achieved. Burst release of Itraconazole from the developed carrier system was found. Itraconazole-loaded NLC possessed good storage stability. Nebulizing Itraconazole-loaded NLC with a jet stream and an ultrasonic nebulizer had no influence on the particle size and the entrapment efficiency of Itraconazole in the particle matrix, being a precondition for pulmonary application.

1. Introduction

Itraconazole is a triazole compound with a broad antifungal spectrum. By interfering with the demethylation of lanosterol to ergosterol by the enzyme 14-α-demethylase Itraconazole inhibits the maintenance of the fungal cell membrane. Inactivation of this enzyme leads to an accumulation of 14-α-methylsterols, such as lanosterol and simultaneously to a diminishment of ergosterol, resulting in a decreased fungal cell membrane synthesis and stability (DeBeule, 1995; Mangino and Pappas, 1995). Itraconazole has a broader spectrum of activity against yeasts, mildews and dermatophytes than other azoles (DeBeule, 1995). Examples for Itraconazole sensitive fungi are *Candida* spp., *Aspergillus* spp., *Penicillium* spp., *Histoplasma capsulatum* and *Blastomyces dermatitidis* (DeBeule, 1995; Mangino and Pappas, 1995).

Opportunistic fungal infections such as aspergillosis, histoplasmosis, blastomycosis or candidiasis can be life-threatening for immuno-compromised patients. The lung as a major port of entry into the body and often site of infection plays an important role in these diseases (Marr et al., 2002). Therefore, the pulmonary application of Itraconazole seems to be a promising therapeutic strategy especially since (I) the lung epithelium can be directly reached resulting in a faster onset of action, (II) the necessary Itraconazole dose and dosing frequency can be reduced compared to traditional administration routs such as oral application and (III) undesirable side effects of Itraconazole such as nausea, abdominal/epigastric pain and hepatotoxicity can be avoided (DeBeule, 1995; Mangino and Pappas, 1995).

Itraconazole is a very poorly water soluble weak base, possessing an aqueous solubility of approx. 1 ng/ml at neutral pH and approx. 4 μg/ml at pH 1 (Jung et al., 1999; Peeters et al., 2002). The n-octanol/water partition coefficient of Itraconazole is 5.66 at a pH of 8.1. Despite the high lipophilicity, resulting in a high cell membrane permeability its poor aqueous solubility makes the development of a sufficiently bioavailable Itraconazole formulation a challenge.

Therefore, in the present study Itraconazole-loaded Nanostructured Lipid Carriers (NLC) were developed. NLC are a nanoparticulate carrier system derived from parenteral o/w emulsions. In NLC the oil component of the o/w emulsion is replaced by a blend of a solid lipid and an oil leading to a solid particle matrix of this carrier system at body temperature (Jenning et al., 2000; Jores et al., 2004). To obtain the blends for the particles matrix, solid lipids are mixed with liquid lipids (oils), preferably in a ratio of 70:30 up to a ratio of 99.9:0.1. The overall solid content of NLC can be up to 95% (w/w) (Müller et al., 1999). The incorporation of

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drugs into lipid nanoparticles is feasible. The mean particle size of the carrier system is in the submicron range, ranging from about 40 nm to 1000 nm (Lucks and Müller, 1991).

Lipid nanoparticles are a carrier system with a number of desirable features: (I) low toxicity (Weyhers et al., 2006), (II) their particulate matrix is easily biodegraded resulting in non-toxic degradation products (Müller and Olbrich, 1999; Olbrich and Müller, 1999), (III) the ability to incorporate lipophilic and hydrophilic drugs (Müller et al., 2002a), (IV) the ability of controlled release of incorporated drugs (Müller et al., 2000b), (V) the ability to immobilize the drug in the solid particle matrix yielding in protection of the incorporated drug from degradation (Jenning and Gohla, 2001; Teeranachaideekul et al., 2007), (VI) easy scale up and manufacture by high pressure homogenization (Müller et al., 2000a), (VI) low cost of excipients, i.e. lipids and stabilizers.

With regard to a pulmonary application NLC also possess several advantages. Applying NLC pulmonary the lower respiratory tract can be reached as reducing the particle size below 500 nm leads to an increased deposition in all lung regions due to an increased diffusional mobility (Jaques and Kim, 2000). Bioadhesive properties of NLC due to their small particle size as well as their lipophilic character lead to longer residence time in the lung. Moreover, it was shown that particles smaller than 260 nm can escape macrophagal clearance (Lauweryns and Baert, 1977). Phagocytic activity is maximal for particles with a diameter of 1–2 μm. Nanomaterials remain longer in the lung fluid. Another important factor is that nanoparticulate formulations compared to microparticulate suspensions show a solution like rheology resulting in a smaller and more uniform droplet size generated during nebulization and therefore a better dose uniformity (Chattopadhyay et al., 2007). Furthermore, controlled release properties of drugs from the solid particle matrix can prolong the therapeutic effect as well as the inhalation interval (Pandey and Khuller, 2005; Patlolla et al., 2010).

The formulation development, especially if a nanoparticulate carrier system should be used, for pulmonary application is quite challenging (Pilcer and Amighi, 2010). In the present study an Itraconazole-loaded NLC formulation which can be administered by nebulization was developed. The formulation development is described including lipid selection, stabilizer selection, optimization of stabilizer concentration, adjustment of tonicity and sterilization. Moreover, Itraconazole-loaded NLC were characterized and their stability investigated. For aerosol application two major types of nebulizers are available on the market, i.e. jet stream nebulizers and ultrasonic nebulizers. Hence, the stability of Itraconazole-loaded NLC with regards to particle size and entrapment efficiency (E.E.) of Itraconazole in the lipid matrix was evaluated after nebulization using the jet stream nebulizer Pari Boy Junior and the ultrasonic nebulizer Beurer IH50.

2. Materials and methods

2.1. Materials

Itraconazole was a gift from Nosch Labs (India). The solid lipids and liquid lipids (oils) used during the experiments were Precirol ATO 5 (Gattefossé, Germany), Compritol 888 ATO (Gattefossé, Germany), Cutina CP (Cognis, Germany), Dynasan 114 (Sasol, Germany), palmic acid (Merck, Germany), stearic acid (Merck, Germany), Witepsol E 85 (Sasol, Germany), Cetiol (Cognis, Germany), almond oil (Henry Lamotte Oils, Germany), Mygliol 812 N (Sasol, Germany), olive oil (Glatter-Koller, Austria), Speziol EOL (Cognis, Germany), Super Refined Corn NF-NP (Croda, Germany), Super Refined Oleic Acid NF (Croda, Germany), Super Refined Peanut NF (Croda, Germany), Super Refined Sesame NF (Croda, Germany), Super Refined Soybean USP-LQ-(MH) (Croda, Germany), Tegosoft M (Evonik, Germany) and Tegosoft P (Evonik, Germany). As potential stabilizer for NLC the following surfactants were under evaluation: Cremophor EL (BASF, Germany), Cremophor RH 40 (BASF, Germany), Eumulgin SLM 20 (Cognis, Germany), Lutrol F68 (BASF, Germany), Span 85 (Merck, Germany) and Speziol TPGS Pharma (Cognis, Germany). As isotonisation agent glycerol 85% (Herba Chemosan, Austria) was used.

2.2. Lipid screening

To evaluate the solid lipid and the liquid lipid (oil) suitable for pulmonary application which dissolves the highest concentration of Itraconazole, increasing concentrations of Itraconazole were added to the lipids and agitated for 2 h at 85 °C with 550 rpm using a Thermomixer comfort (Eppendorf, Germany). Furthermore, the miscibility of the solid lipid and the liquid lipid as well as the solubility of Itraconazole in the mixture of the lipids with the best solubility of Itraconazole was evaluated under the same conditions.

2.3. Polarized light microscopy

To obtain information if recrystallisation of Itraconazole from the solid lipids, the bulk lipid mixture and the NLC takes place, the samples were investigated using polarized light microscopy (Axiovert 40 CFL, Carl Zeiss, Germany) using a 200-times magnification.

2.4. Differential scanning calorimetry

The melting behavior of Itraconazole, Precirol ATO 5, bulk mixture of Precirol ATO 5 and oleic acid (9:1), ternary bulk mixture of Itraconazole, Precirol ATO 5 and oleic acid (1:225:25) and Itraconazole-loaded NLC was studied using a DSC 204 F1 Phoenix (Netzsch, Germany). Precirol ATO 5 and the bulk mixtures were tempered for 1 h at 60 °C to mimic production conditions of NLC. 2–4 mg Precirol ATO 5 and bulk mixtures were analyzed in sealed aluminium pans. An empty aluminium pan was used as a reference. Samples were heated from 20 °C to 200 °C with a heating rate of 5 K/min under constant flushing with nitrogen (80 ml/min). To evaluate the melting point of NLC 20–40 mg of the samples were weight in aluminium pans, heated from 20 °C to 80 °C with a heating rate of 5 K/min under constant flushing with nitrogen (80 ml/min).

2.5. X-ray

X-ray diffraction $\theta/2\theta$ measurements were performed using a Siemens D-501 (Siemens, Germany) (coupled $\theta/2\theta$) diffractometer in Bragg–Brentano geometry. CuKα radiation was used in combination with a secondary graphite monochromator.

2.6. Contact angle measurements

Contact angles were determined by goniometry on cover slides coated with a thin film of the bulk mixture of Precirol ATO 5:oleic acid (9:1). 10 μl of purified water or 0.1% (w/v) surfactant/stabilizer solution in purified water, respectively, was applied to the lipid film. The contact angle was measured using an Easy Drop G1 (Krüss, Germany). The experiment was carried out in triplicate.

2.7. Preparation of NLC

NLC were prepared by hot high pressure homogenization using a Panda K2 NS1001L Spezial modified for NLC production (GEA Niro Soavi, Germany). Briefly, Itraconazole was dissolved in a mixture of the solid lipid and the liquid lipid at 60 °C. Eumulgin SLM 20 and glycerol 85% were dissolved in water for injection at 60 °C.

The aqueous phase was added to the oil phase and stirred by high speed stirring (8000 rpm) using an Ultra Turrax T25 (IKA-Werke, Germany). The obtained pre-emulsion was subjected to high pressure homogenization applying 3 cycles at 800 bar and 60 °C.

2.8. Photon correlation spectroscopy

The particle size of Itraconazole-loaded NLC was analyzed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, UK) equipped with a green laser. PCS yields the mean particle size and the polydispersity index (PI) as a measure of the width of the particle size distribution.

2.9. Laser diffraction

The presence of particles in the micrometer range in the NLC formulation was excluded by laser diffraction (LD) using a Mastersizer 2000 (Malvern Instruments, UK). For LD data evaluation the Mie theory was used. Water with a refractive index of 1.33 was used as measurement medium. The real refractive index and the imaginary refractive index were set 1.456 and 0.01, respectively. The diameter 50% (LD 50), 90% (LD 90), 95% (LD 95) and 99% (LD 99), which means that 50%, 90%, 95% or 99% (volume distribution) of the measured particles are below this size, were evaluated.

2.10. Zeta potential

The zeta potential of Itraconazole-loaded NLC was determined via electrophoretic mobility measurements using a Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed in distilled water adjusted with 0.9% (w/v) sodium chloride solution to a conductivity of 50 $\mu S\,cm^{-1}$ and a pH of 5.5–6.0. The zeta potential was calculated applying the Helmholtz–Smoluchowski equation ($n = 3$).

2.11. Osmolarity measurements

The osmolarity of Itraconazole-loaded NLC was measured using an Osmomat 030-D (Gonotec, Germany).

2.12. Autoclaving

To ensure sterility of the formulation, Itraconazole-loaded NLC were autoclaved (121 °C, 15 min) using an ASB 030 (Astell Scientific, UK).

2.13. Entrapment efficiency

The entrapment efficiency (E.E.) of Itraconazole into NLC was determined by ultrafiltration method using centrifugal filter tubes with a molecular weight cut-off of 30 kDa (Amicon Ultra, Millipore, Ireland). The concentration of Itraconazole in NLC and in the ultrafiltrate (free drug) was analyzed using HPLC. The E.E. was calculated using the following equation:

$$\text{E.E.} = \frac{\text{Total amount of Itraconazole} - \text{Amount of free Itraconazole}}{\text{Total amount of Itraconazole}} \quad (1)$$

2.14. HPLC method

To quantify Itraconazole an HPLC system composed of an autosampler model Merck-Hitachi L-7200, a pump system model Merck-Hitachi L-6200 Intelligent Pump and an UV/Vis detector Merck-Hitachi L-4250 (Merck, Germany) linked to a D-7000 HSM data acquisition and process system was used. 20 μl of the sample was injected onto a YMC-Pack ODS-AQ RP-18 (5 μm) endcapped 250 mm × 4 mm column (YMC Europe, Germany) which was kept at 30 °C. As solvent for sample preparation a mixture of dichloromethane (Carl Roth, Germany) and dimethylsulfoxide (Sigma–Aldrich, Germany) in the ratio 4:1 was used. The mobile phase, which was run with a flow rate of 1 ml/min, was a mixture of methanol (Merck, Germany) and MilliQ water in the ratio 75:25. The UV-spectrum was recorded at 267 nm. The HPLC method was validated over a range from 2.5 μg/ml to 250 μg/ml. The limit of detection and limit of quantification were 0.6 μg/ml and 2 μg/ml, respectively.

2.15. SEM

Scanning electron microscopy (SEM) was performed at the Institute for Electron Microscopy (Graz, Austria) using a ZEISS Ultra 55 (Carl Zeiss SMT GmbH, Germany). Prior to analysis, samples were diluted with MilliQ water, dropped on an amorphous carbon grid, air-dried at room temperature and coated with chrome.

2.16. Release study

The release of Itraconazole from NLC was studied under sink conditions using an aqueous solution containing 20% (w/w) 2-hydroxypropyl-beta cyclodextrin (Wacker, Germany) as dissolution medium. Briefly, NLC were placed in the dissolution medium obtaining a total Itraconazole concentration of 27.5 μg/ml. After shaking the samples for 5, 10, 20, 30, 60 and 240 min at 37 °C with 500 rpm using a Thermomixer comfort (Eppendorf, Germany) the samples were filtrated using 0.02 μm filters (Anotop 25 Plus, Whatman, UK). The amount of Itraconazole released from NLC was evaluated by HPLC. The experiment was carried out in triplicate.

2.17. Nebulization of Itraconazole-loaded NLC

Itraconazole-loaded NLC were nebulized using the jet stream nebulizer Pari Boy Junior (Pari, Germany) and the ultrasonic nebulizer Beurer IH50 (Beurer, Germany). The particle size of Itraconazole-loaded NLC was measured by PCS before nebulization and after collection of the aerosol using the inhalation test apparatus from glass according to the European Pharmacopeia (2007) ($n = 5$). Furthermore, the E.E. of Itraconazole in NLC was evaluated before and after nebulization with both devices. For statistical evaluation the two sample t-test for unpaired data was performed after testing for normal distribution using the Shapiro–Wilk test ($\alpha = 5\%$).

3. Results and discussion

3.1. Selection of matrix lipids

A large variety of solid lipids and liquid lipids including natural, semi-synthetic and synthetic lipids with various structures, e.g. triglycerides, partial glycerides, fatty acids, waxes and steroids are available as matrix lipids for NLC production. However, the lipids used as matrix lipids need to be carefully selected as they will directly influence the performance of the carrier system. Properties directly influenced by the lipids selected are (I) the toxicity and biocompatibility by selecting well tolerated physiological and biodegradable lipid, (II) the drug payload and entrapment efficiency by choosing lipids in which the drug shows a high solubility and which possess a low crystallinity, (III) drug expulsion during storage which can be minimized or avoided with lipid matrices with a low tendency of crystallization or a less ordered structure, (IV) controlled drug release properties via the way of incorporation

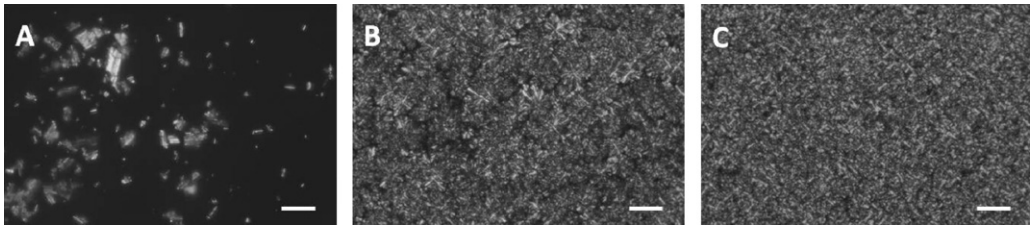

Fig. 1. Polarized light microscope images of Itraconazole crystals (A), lipid blend of Precirol ATO 5:oleic acid 9:1 (B) and lipid blend containing 0.4% (w/w) Itraconazole (C). The bar refers to 10 μm.

Table 1
Overview of solid lipids and liquids lipids and their ability to dissolve Itraconazole (+, dissolved, (+), dissolved in heat but recrystallization at room temperature, –, not dissolved).

	Lipid	Itraconazole content [%]		
		0.5	0.4	0.3
Solid lipids	Compritol 888 ATO	–	–	–
	Cutina CP	–	–	–
	Dynasan 114	–	–	–
	Palmic acid	–	–	–
	Precirol ATO 5	–	+	+
	Stearic acid	–	–	–
	Witepsol E 85	–	–	–
Liquid lipids	Almond oil	–	–	–
	Cetiol	–	–	–
	Corn oil	–	–	–
	Mygliol 812 N	–	–	–
	Oleic acid	(+)	+	+
	Olive oil	–	–	–
	Peanut oil	–	–	–
	Sesame oil	–	–	–
	Soybean oil	–	–	–
	Speziol EOL NF	–	–	–
	Tegosoft M	–	–	–
	Tegosoft P	–	–	–

of the drug into the lipid matrix (i.e. drug-enriched core, drug-enriched shell or homogeneous drug distribution in the matrix) and (V) increased chemical drug stability in case photosensitive drugs, drugs sensitive to hydrolysis or oxidation are incorporated in the matrix (Mehnert and Mäder, 2001; Müller et al., 2002b; Pardeike et al., 2009).

To find the solid lipids and liquid lipids which can dissolve the highest concentration of Itraconazole and therefore maximize the Itraconazole payload and E.E., a lipid screening was performed using solid lipids and liquid lipids which are well tolerated after pulmonary application. The lipids chosen for the evaluation as well as their potential to dissolve Itraconazole are shown in Table 1. By visual evaluation and polarized light microscopy it was found that the highest amount of Itraconazole could be dissolved in the solid lipid Precirol ATO 5 as well as in the liquid lipid oleic acid.

Blending a solid lipid with a liquid lipid leads to a less ordered solid lipid matrix providing the possibility for a high drug payload (Hu et al., 2006; Müller et al., 2002a,b). To avoid the formation of oil droplets during high pressure homogenization and therefore the co-existence of NLC and o/w emulsion, the liquid lipid and the solid lipid need to be well mixable in the ratio used for lipid matrix production. Precirol ATO 5 and oleic acid were well mixable in the ratio 9:1. No oil expulsion from the solid lipid blend was observed.

Furthermore, the lipid blend was able to dissolve 0.4% (w/w) Itraconazole. Fig. 1 shows polarized light microscope images of Itraconazole crystals, the pure lipid blend and the lipid blend containing 0.4% (w/w) Itraconazole. No Itraconazole crystals were found in the lipid blend indicating a well solubility of the drug in the lipid mixture.

The DSC thermograms of Itraconazole, Precirol ATO 5, bulk mixture of Precirol ATO 5 and oleic acid (9:1) and ternary bulk mixture of Itraconazole, Precirol ATO 5 and oleic acid (1:225:25) are shown in Fig. 2. Itraconazole showed a melting peak at 168.5 °C indicating the crystalline state of the drug. Precirol ATO 5 as well as the bulk mixtures were heated up to 60 °C and kept at that temperature for one hr to mimic production conditions of NLC. The DSC curves were recorded 1 day after tempering of the materials. Two modifications of Precirol ATO 5 melting at 51 °C and 58 °C were found. The tempered bulk mixture of Precirol ATO 5 and oleic acid in a ratio 9:1, which is the same ratio used in Itraconazole-loaded NLC, was investigated by DSC in order to obtain further information on the inclusion of the liquid lipid in the solid lipid and the influence on the melting behavior. The less stable modification of Precirol ATO 5 could not be detected in the bulk mixture. Furthermore, a melting point depression of the stable modification to 53 °C and widening of the melting peak was observed compared to Precirol ATO 5, which indicates that oleic acid is dissolved in Precirol ATO 5, inducing a less pronounced crystalline structure. In the next step the tempered ternary mixture of Itraconazole, Precirol ATO 5 and oleic acid in the ratio used in Itraconazole-loaded NLC was investigated in order to obtain information about the ability of the lipid matrix to dissolve Itraconazole. In this mixture no melting peak for Itraconazole was detected indicating that Itraconazole was dissolved in the lipid blend used as particle matrix for NLC production.

The XRD $\theta/2\theta$ scans of Itraconazole, the bulk mixture of Precirol ATO 5 and oleic acid (9:1) as well as the ternary mixture of Itraconazole, Precirol ATO 5 and oleic acid (1:225:25) are shown in Fig. 3. The spectrum obtained from Itraconazole powder gives quite sharp and distinct diffraction peaks with a low and flat background as expected from a crystalline sample. The diffraction pattern is in good agreement with the crystal structure determined by Peeters et al. (1996). The mixture of Precirol ATO 5 and oleic acid shows some distinct peaks as well, but the higher background in the range of 15°–25° indicates a lower crystallinity, i.e. an amorphous fraction. However, the mixture of both is decisively different. Though the features of the Precirol:oleic acid mixture are broadening, most of them can still be recognized in the ternary system. In contrary, no traces from the crystalline Itraconazole phase are observed. Since the low fraction of Itraconazole in the ternary mixture is close to the detection limit of the presented measurements more precise measurements at selected diffraction angles were performed to reliably exclude the presence of the initial crystal structure as a relevant phase. Furthermore, a new broad feature rises at about $2\theta = 21.4°$ which can be explained neither by the Itraconazole nor by the Precirol:oleic acid mixture initial phases. These results suggest that the crystalline Itraconazole is dissolved and embedded in the bulk mixture of Precirol and oleic acid leading to a molecular arrangement that gives the additional XRD feature. This is well in agreement with the results obtained by polarized light microscopy and DSC. Therefore, it can be concluded that the blend of Precirol ATO 5 and oleic acid in a ratio 9:1 is suitable as lipid matrix for the production of NLC.

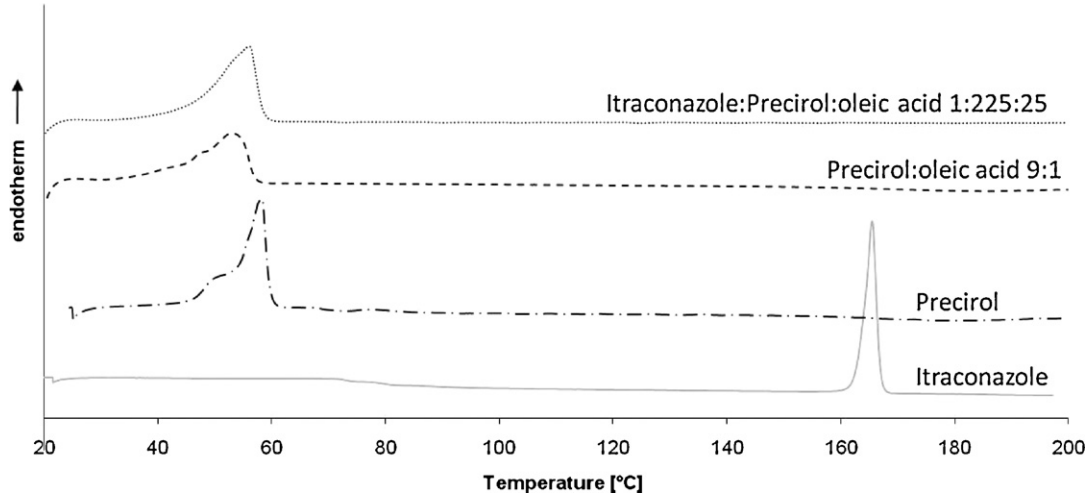

Fig. 2. DSC scans of Itraconazole, Precirol ATO 5, bulk mixture of Precirol ATO 5 and oleic acid (9:1) and ternary bulk mixture of Itraconazole, Precirol ATO 5 and oleic acid (1:225:25).

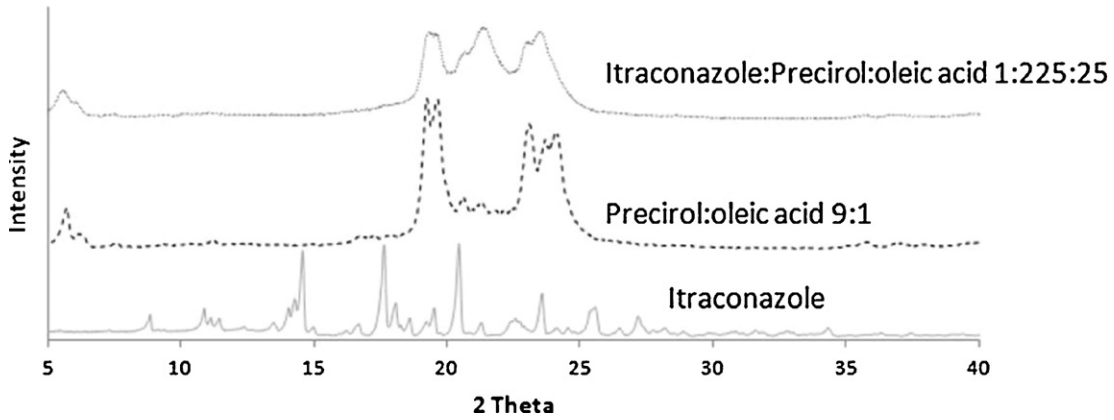

Fig. 3. X-ray diffraction patterns of Itraconazole, bulk mixture of Precirol ATO 5 and oleic acid (9:1) and ternary bulk mixture of Itraconazole, Precirol ATO 5 and oleic acid (1:225:25).

3.2. Selection of the stabilizer and stabilizer concentration

As the stabilizer/surfactant reduces the interfacial tension between the lipid matrix and the water phase of the lipid nanoparticles, the nature and concentration of the stabilizer is a highly important factor for the fineness and physical long-term stability of lipid nanoparticles (Üner et al., 2004). A good measure for the interfacial tension is the contact angle. The lower the interfacial tension between the lipid matrix and the aqueous dispersion medium, the smaller is the contact angle between them. Therefore, to evaluate the stabilizer/surfactant which shows the best wetting of the Precirol ATO 5:oleic acid 9:1 mixture, contact angle measurements were performed. The results are shown in Table 2. With all stabilizer/surfactant solutions smaller contact angles were obtained on the matrix lipid mixture than with purified water. This is due to the fact that surfactants/stabilizers exhibit a smaller surface tension than purified water. Eumulgin SLM 20 was the surfactant which showed the best wetting of the matrix lipid mixture and was therefore used as stabilizer for NLC production.

Table 2
Contact angles obtained with water and 0.1% (w/v) stabilizer solutions on lipid films of Precirol ATO 5 and oleic acid in a ratio of 9:1.

Stabilizer	Contact angle (°)
Water	108.67 ± 1.53
Cremophor EL	90.67 ± 1.53
Cremophor RH 40	88.83 ± 1.76
Eumulgin SML 20	79.67 ± 1.16
Lutrol F 68	84.00 ± 0.00
Span 85	105.83 ± 1.04
Tego Care 450	110.83 ± 2.36
Speziol TPGS Pharma	86.33 ± 0.58

The surfactant concentration has a great impact on the particle size distribution, the stability and the toxicological potential of lipid nanoparticles (Han et al., 2008; Scholer et al., 2001; Üner et al., 2004). A high surfactant concentration favors a lower particle size, a narrower particle size distribution and a better long-term stability of lipid nanoparticles but simultaneously increases the toxicological potential. Hence, a balance needs to be found to have sufficient surfactant present to ensure a small particle size and a good physical stability of the carrier system as well as avoiding free surfactant in the formulation as binding surfactants to the surface of lipid nanoparticles markedly reduces the toxicological potential. In the present study the lipid content of the Itraconazole-loaded NLC was set 5% (w/w). The overall Itraconazole-content was 0.02% (w/w). The influence of an increasing Eumulgin SLM 20 concentrations ranging from 0.5% (w/w) to 3% (w/w) on the particle size and physical stability was studied. Fig. 4 shows the correlation between the Eumulgin SLM 20 concentration and the particle size of the obtained NLC measured by PCS. Itraconazole-loaded NLC stabilized with 0.5% Eumulgin SLM 20 possessed a high agglomeration tendency. The particles stabilized with 1% Eumulgin SLM 20 were also

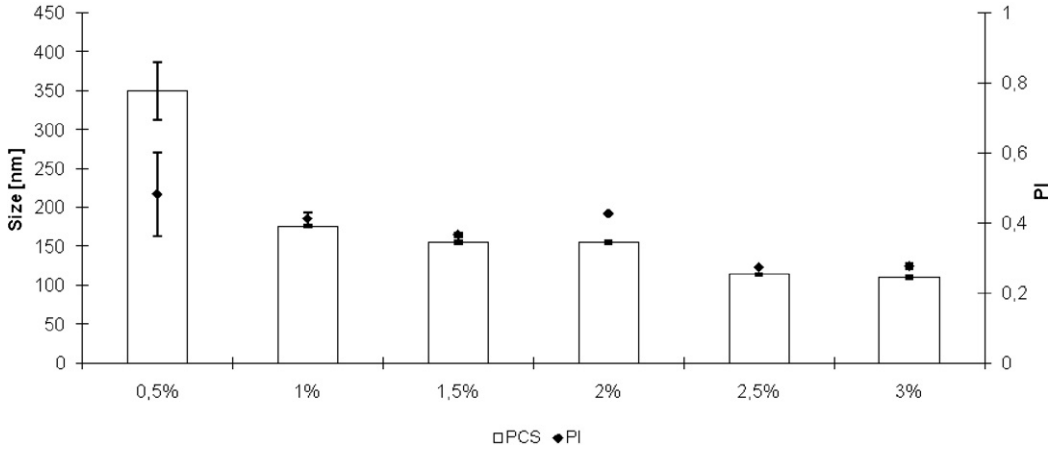

Fig. 4. Correlation between the particle sizes of Itraconazole-loaded NLC measured by PCS and the Eumulgin SLM 20 concentration used for stabilization.

instable. For this formulation an increase of the average particle size from 177 nm at the day of production to 208 nm at day 1 was found. The particle size of Itraconazole-loaded NLC stabilized with 1.5% and 2% Eumulgin SLM 20 was similar. However, both formulations showed a PI indicating a broad particle size distribution favoring physical instabilities such as particle growth. For both formulations an increase in particle size was found within 3 weeks. Increasing the Eumulgin SLM 20 concentration to 2.5% and 3% resulted in a further decrease in particle size and PI. No difference was found in the particle size distribution of these formulations. Furthermore, both formulations were physical stable over an observation period of 3 weeks. As 3% Eumulgin SLM 20 had no advantage with regard to the particle size distribution and physical stability it was further proceeded in formulation development with the Itraconazole-loaded NLC formulation stabilized with 2.5% Eumulgin SLM 20.

3.3. Tonicity, sterility and pH value

Pulmonary application requires formulations being isotonic, having a pH value between 3 and 8.5 and being sterile (2007). Itraconazole-loaded NLC were produced using water for injection. Table 3 shows the osmolarity, pH value, particle size and the zeta potential measured for the formulation. An osmolarity of 0.012 osmol/kg was measured, indicating that the osmolarity needs to be adjusted to obtain an isotonic formulation for pulmonary application. It is well known that instabilities of dispersed systems like NLC might occur if electrolytes such as sodium chloride or potassium chloride are added in sufficient high concentrations that the formulation becomes isotonic. The addition of salts to the formulation would result in compression of the diffuse layer resulting in a reduction of the zeta potential and therefore electrostatic destabilization (Müller, 1996). Hence, electrolytes cannot be used to increase the osmotic pressure as destabilization of the formulation should be avoided. Alternatively non-ionic substances such as glycerol or carbohydrates might be used. In the present study glycerol 85% was added as isotonisation agent to the aqueous phase of Itraconazole-loaded NLC prior to high pressure homogenization. The obtained osmolarity, pH value, particle size and the zeta potential after addition of glycerol 85% to Itraconazole-loaded NLC are shown in Table 3. Neither the particle size nor the zeta potential was influenced by the addition of glycerol 85%, indicating the suitability of glycerol 85% as isotonisation agent for Itraconazole-loaded NLC. The absolute zeta potential value of the colloidal system was above 30 mV. As Itraconazole-loaded NLC have been stabilized with Eumulgin SLM 20, which means that the physical stability has been enhanced not only by electrostatic stabilization but also by steric hindrance of surfactant chains, good physical long term stability can be assumed for the carrier system (Jacobs and Müller, 2002).

Heat, γ-irradiation, aseptic processing and filtration through microbial retentive materials are usually used to obtain sterile formulations. Autoclaving is a simple method being advantageous compared to aseptic processing to obtain sterility of nanoparticulate carrier systems with heat resistant drugs. However, autoclaving might result in heat-induced changes in physical stability. The temperature reached during autoclaving causes the lipid particle matrix to melt. Via the high energy input coalescence of the oil droplets of the obtained o/w emulsion might occur resulting in an increase in particle size of lipid nanoparticles (Mehnert and Mäder, 2001; Schwarz et al., 1994). Table 3 shows the particle size, zeta potential, osmolarity and pH value of the autoclaved isotonic Itraconazole-loaded NLC formulation. The particle size of the formulation was not affected by autoclaving. Neither by PCS nor by LD measurements changes in the particle size were found after autoclaving. Moreover, no changes in zeta potential were observed indicating a good stability of the sterile formulation. These results are well in agreement with earlier reports, where a good stability of lipid nanoparticles subjected to autoclaving was shown (Cavalli et al., 1997; Kuntsche and Bunjes, 2007).

The pH value of Itraconazole-loaded NLC was well in the pH range required for pulmonary application before isotonisation (4.36), after isotonisation (4.07) and after sterilization (4.05) of Itraconazole-loaded NLC. The developed formulation fulfills the requirements of the European Pharmacopeia for pulmonary applied solution with regards to isotonicity, pH value and sterility.

3.4. Characterization of Itraconazole-loaded NLC

To obtain more information about the particle size and the shape of Itraconazole-loaded NLC SEM analysis was performed. Fig. 5 shows the LD volume distribution curve and a SEM image of the lipid nanoparticles. SEM revealed a spherical shape of the particles with a homogeneous particle size distribution. Moreover, the diameter of the particles observed in the SEM image is in good agreement with PCS and LD measurements (Fig. 3).

The E.E. of Itraconazole in NLC was calculated using Eq. (1). The E.E. of Itraconazole in NLC was 98.78%. Moreover, drug crystals could not be observed by polarized light microscopy, indicating incorporation of Itraconazole in the lipid matrix of NLC. These results can be explained via the high affinity of the lipophilic drug Itraconazole to Precirol ATO 5 and oleic acid as well as by its low aqueous solubility (Peeters et al., 2002). Furthermore, the incorporation of the liquid lipid oleic acid in the solid lipid Precirol ATO 5 lead to a massive crystal order disturbance, resulting in a lipid

Table 3
Overview of particle size measured by PCS and LD, zeta potential (ZP), osmolarity and pH value of Itraconazole-loaded NLC before isotonisation, after isotonisation and after autoclaving of the isotonic formulation.

Measuring parameter	Before isotonisation	After isotonisation	After sterilization
PCS [nm]	114	103	106
PI	0.273	0.230	0.202
LD 50 [μm]	0.133	0.130	0.130
LD 90 [μm]	0.195	0.191	0.190
LD 95 [μm]	0.215	0.210	0.209
LD 99 [μm]	0.251	0.246	0.244
ZP [mV]	−33.3 ± 0.4	−34.2 ± 1.0	−32.7 ± 0.7
Osmolarity [osmol/kg]	0.012 ± 0.001	0.288 ± 0.011	0.269 ± 0.002
pH value	4.36 ± 0.04	4.07 ± 0.16	4.05 ± 0.03

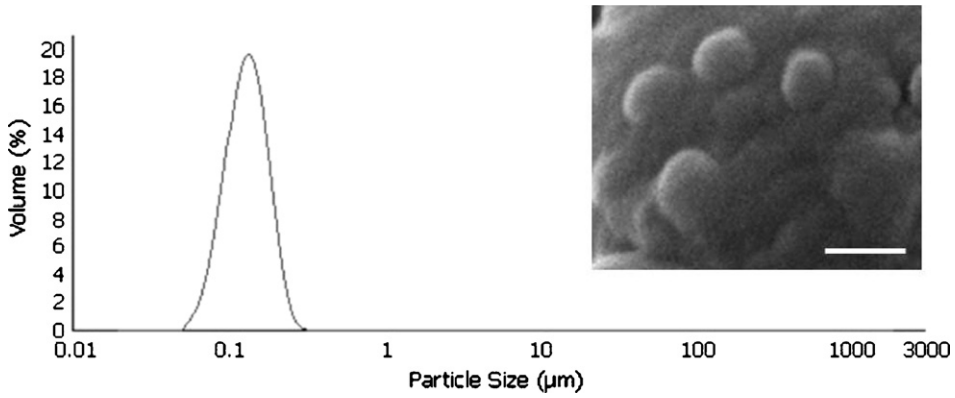

Fig. 5. LD volume distribution curve and SEM image of Itraconazole-loaded NLC. The bar refers to 100 nm.

matrix with imperfections in the crystal lattice and therefore providing enough space to accommodate drug molecules in the matrix leading to a high E.E. (Hu et al., 2006; Jenning et al., 2000).

The melting behavior of the sterile and isotonic formulation was investigated by DSC. Itraconazole-loaded NLC showed an onset at 46.1 °C and a melting point of 50.9 °C. Due to the presence of a melting point the formation of a supercooled melt can be excluded. To retain the particle matrix of the Itraconazole-loaded NLC in a solid state after pulmonary application, the melting point of the particle matrix should be above body temperature, i.e. 37 °C. The found onset and melting point of Itraconazole-loaded NLC indicate the presence of a solid particle matrix of the carrier system at body temperature. The melting point obtained for the NLC formulation was about 2 K lower than the one of the tempered bulk mixtures. In addition to a possible effect of the surfactant used to stabilize the NLC dispersion, this can be explained by the small particle size and the high specific surface area of NLC according to the Gibbs–Thomson equation (Bunjes and Unruh, 2007; Saupe et al., 2005).

A precondition to obtain a pharmaceutical effect is that the applied drug delivery system is able to release the drug at the side of action or the absorption side, respectively. For lipid nanoparticles a fast release (e.g. 100% release in less than 1 min), sustained release (e.g. over 5 weeks) as well as an initial burst release followed by prolonged release have been reported (Hu et al., 2006; Zhang et al., 2010; zur Mühlen et al., 1998; Mitiri et al., 2011). A solid particle matrix is a precondition to adjust the release profile of a drug from lipid nanoparticles as the release from o/w emulsions is very fast and takes place within seconds (Benita et al., 1986). Fig. 6 shows the release profile of Itraconazole-loaded NLC. The developed carrier system showed burst release, i.e. 80% Itraconazole release within 5 min. It can be excluded that the formation of drug crystals is responsible for the fast release as Itraconazole possessed high E.E. in the lipid particle matrix and no drug crystals were observed by polarized light microscopy. A possible explanation for the observed burst release is the large surface area of Itraconazole-loaded NLC as well as a short diffusion distance for Itraconazole from the particle matrix into the dissolution medium due to an enrichment of the drug in the outer region of the NLC (Müller et al., 2002b; Souto et al., 2004; zur Mühlen et al., 1998). However, the developed carrier system is able to release Itraconazole in vitro being a precondition for a therapeutical effect in vivo.

3.5. Stability investigations

In order to evaluate the physical stability Itraconazole-loaded NLC were stored at two different temperatures, i.e. room temperature and refrigerated, and the particle size, the zeta potential and the E.E. was measured over a period of 6 months. Figs. 7 and 8

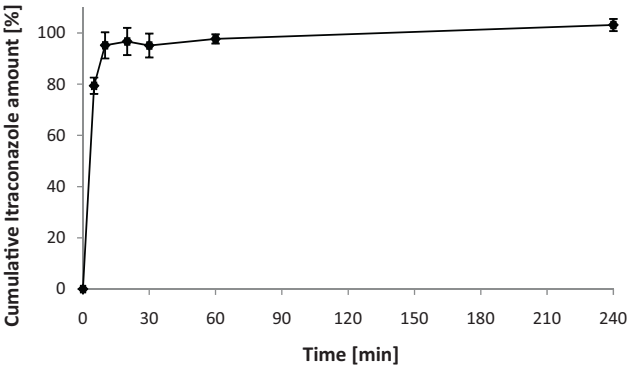

Fig. 6. In vitro release profile of Itraconazole-loaded NLC.

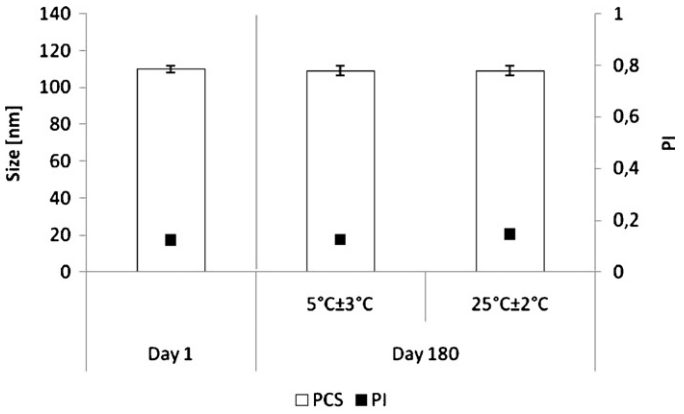

Fig. 7. Average particle size and PI of Itraconazole-loaded NLC at the day of production and after 180 days of storage at 5 ± 3 °C and 25 ± 2 °C.

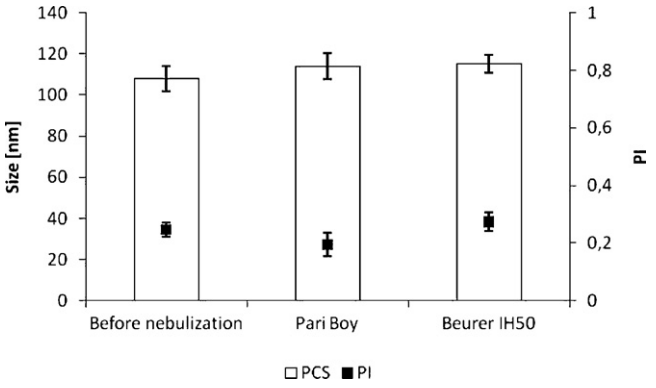

Fig. 9. Average particle size and PI of Itraconazole-loaded NLC before nebulization and after nebulization using a Pari Boy Junior jet stream nebulizer and a Beuer IH50 ultrasonic nebulizer ($n = 5$).

show the particle size of Itraconazole-loaded NLC measured by PCS and LD at the day of production and after 6-month of storage. Neither by PCS nor by LD measurements particle growth was found if the carrier system was stored at room temperature or refrigerated. The zeta potential was −32.7 ± 0.7 mV at the day of production. After 6-month of storage at room temperature and refrigerated a zeta potential of −31.3 ± 0.7 mV and −31.6 ± 2.5 mV was measured, respectively. The zeta potential stayed unchanged over the observation period at both storage temperatures. As instabilities such as aggregation or agglomeration as well as gelation phenomena of lipid nanoparticles are indicated by a decrease of the absolute zeta potential value, the particle size of Itraconazole-loaded NLC is expected to stay unchanged beyond the observation period (Freitas and Müller, 1998). A physical stability of lipid nanoparticles up to 3 years has previously been reported (Freitas and Müller, 1998).

The E.E. stayed unchanged over the observation period of 6-month at both storage temperatures, providing evidence that Itraconazole is enclosed in the lipid particle matrix. Due to mixing the solid lipid Precirol ATO 5 with the liquid lipid oleic acid imperfections in the particle matrix were created providing spaces for the accommodation of the drug. However, Kim et al. found a lower E.E. as well as a reduction of E.E. during storage of Itraconazole-loaded NLC prepared from the solid lipid tristearin and the liquid lipid triolein (Kim et al., 2010). Tristearin and triolein are triglycerides of C18 fatty acids, i.e. stearic acid and oleic acid. In the present study Precirol ATO 5, a mixture of mono-, di- and triglycerides of palmic acid (C16) and stearic acid (C18), and the liquid lipid oleic acid (C18) were used, which showed due to higher structural differences no tendency for drug exclusion from the particle matrix.

3.6. Nebulization of Itraconazole-loaded NLC

Jet stream and ultrasonic nebulizers are the most frequently used nebulizers for inhalation therapy. In nanoparticulate delivery systems instabilities such as aggregation, agglomeration, disruption of nanocarriers as well as loss of the encapsulated drug can occur since the particles are subjected to shear stress and additionally to an increase in temperature during aerosolization using ultrasonic nebulizers (Rudolph et al., 2004; Taylor et al., 1990). Therefore, in this study the effect on particle size and encapsulation efficiency of Itraconazole-loaded NLC was studied nebulizing the formulation with a jet stream nebulizer, i.e. Pari Boy Junior and an ultrasonic nebulizer, i.e. Beurer IH50. Fig. 9 shows the average particle size and PI of Itraconazole-loaded NLC before and after nebulization using a jet stream and an ultrasonic nebulizer. Before nebulization the NLC formulation had an average particle size and PI of 108 nm and 0.247, respectively. A particle size and PI of 114 nm and 0.194 was measured after nebulization using Pari Boy Junior. Nebulization with Beurer IH50 ultrasonic nebulizer leads to an average particle size of 115 nm and a PI of 0.274. There were no significant changes in particle sizes nebulizing Itraconazole-loaded NLC using a jet stream and an ultrasonic nebulizer. Neither particle agglomeration/aggregation nor fragmentation of the lipid nanoparticles took place. The E.E. of Itraconazole in NLC was 98.78 ± 0.86%.

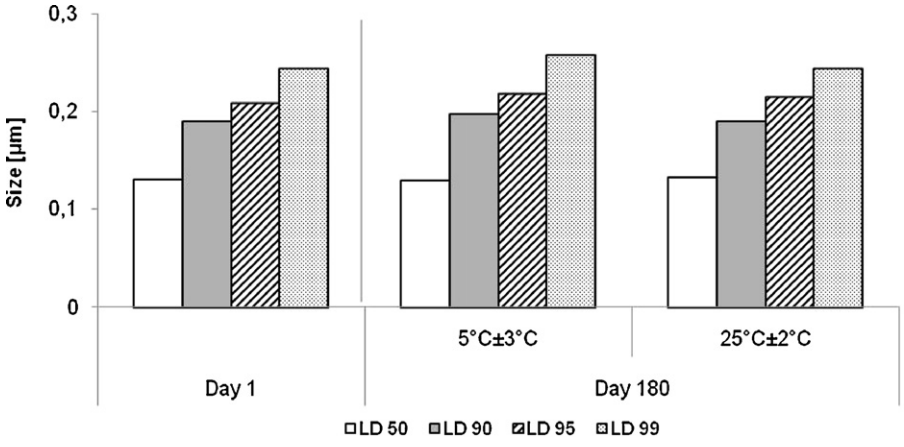

Fig. 8. LD 50, LD 90, LD 95 and LD 99 of Itraconazole-loaded NLC at the day of production and after 180 days of storage at 5 ± 3 °C and 25 ± 2 °C.

After nebulization using Pari Boy Junior and Breuer IH50 an E.E. of 98.96 ± 0.23% and 99.22 ± 0.15% was found. There was no significant difference in the E.E. before and after nebulization with both nebulizer types. Hence, the developed Itraconazole-loaded NLC formulation is stable during nebulization via jet stream and ultrasonic nebulization. The obtained results are well in accordance with earlier findings. Hu et al. (2010) as well as Patlolla et al. (2010) previously showed an unchanged particle size and E.E. of lipid nanoparticles subjected to jet stream nebulization. Videira et al. (2002) used an ultrasonic nebulizer for lipid nanoparticle nebulization and showed an unchanged average particle size.

4. Conclusion

Selecting matrix lipids and stabilizers based on solubility and miscibility investigations and contact angle measurements, respectively, can help formulating NLC with good storage stability and a high E.E. as demonstrated in the present study for Itraconazole. Furthermore, NLC are a carrier system with which the requirements of the European Pharmacopeia with regards to tonicity, sterility and pH value can be fulfilled using glycerol as isotonisation agent and subjecting the carrier system to autoclaving. Moreover, Itraconazole-loaded NLC were stable during nebulization using a jet stream and a ultrasonic nebulizer, maintaining their advantageous properties like a small particle size and an high E.E. Itraconazole-loaded NLC are a promising carrier system for the pulmonary application of the poorly soluble triazol drug with its broad antifungal spectrum.

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