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Encapsulation of ascorbyl palmitate in nanostructured lipid carriers (NLC)—Effects of formulation parameters on physicochemical stability

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Abstract

Enhancement of the chemical stability of ascorbyl palmitate (AP) after incorporation into nanostructured lipid carriers (NLC) has been reported. However, the formulation parameters of AP-loaded NLC have not been completely investigated. Moreover, the long-term chemical stability of AP in any colloidal systems has not been yet achieved. Therefore, in this study the formulation parameters affecting the stability of AP after incorporation into NLC were evaluated including types of lipids, types of surfactants, storage conditions, i.e. temperature and nitrogen gas flushing, the effects of drug loading as well as types of antioxidants. After storage for 90 days, the mean particle size analyzed by photon correlation spectroscopy (PCS) was lower than 350 nm. The zeta potential measured by the Zetasizer IV was higher than −30 mV in all developed AP-loaded NLC formulations which varied according to the types of lipid and surfactant. Concerning the chemical stability of AP, it was found that AP-loaded NLC prepared and stored in non-degassing conditions, a higher percentage of AP loading in NLC, lower storage temperature (4 °C), addition of antioxidants as well as selection of suitable surfactants and solid lipids improved the chemical stability of AP. Moreover, an improvement of long-term chemical stability of AP was achieved by addition of antioxidants with nitrogen gas flushing as compared to those without antioxidant. The percentage of drug remaining at both 4 °C and room temperature (25 °C) was higher than 85% during 90 days of storage.

Keywords: Nanostructured lipid carriers; NLC; Ascorbyl palmitate; Chemical stability; Antioxidant

1. Introduction

In recent years, nanotechnology has been intensively studied in many fields such as computer, engineering, electronic as well as pharmaceutical technology. In the pharmaceutical field, several advantages of drug delivery systems with nanosize range have been shown including increasing solubility, enhancing dissolution rate and improving bioavailability (Müller et al., 2000). Nanoparticles can be prepared using different kinds of materials, for example, biodegradable and biocompatible polymers, phospholipids, surfactants and lipids (Müller et al., 2000, 2002b).

Several advantages of nanoparticles prepared from lipid materials have been demonstrated including biocompatibility, drug targeting, modified release, lack of organic solvent during the production process and ease of large scale production (Mehnert and Mäder, 2001; Müller et al., 2000). Moreover, lipid nanoparticles have been intensively studied in various pharmaceutical applications, i.e. parenteral, peroral, dermal, ocular, and pulmonary administrations (Mehnert and Mäder, 2001; Müller et al., 1997, 2000; Pandey et al., 2005; Schwarz and Mehnert, 1997; Sivaramakrishnan et al., 2004; Cavalli et al., 2002). However, it has been reported that the physical state of lipid particles plays a major role in increasing stability of active compound after incorporation into lipid nanoparticles especially solid state lipids. This is due to the fact that the liquid state of colloidal system allows the active ingredients to partition between dispersed and continuous phase (Dingler et al., 1996). This leads to the instability of the active ingredient in the continuous phase during storage times. Therefore, lipid nanoparticles with solid dispersion phase have been introduced as alternative colloidal carriers and referred to as solid lipid nanoparticles (SLN). Since the last decade, they have been studied intensively both in pharmaceutical and cosmetic

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areas because they can be prepared using a simple method, i.e. high pressure homogenization (HPH), which is a simple technique commonly used in food and pharmaceutical industries. Nevertheless, SLN also possess some limitations, i.e. low drug payload and possibility of drug expulsion because of change of the lipid modification during the storage times (Mehnert and Mäder, 2001). As a result, the concept of less ordered inner structure has been introduced, namely nanostructured lipid carriers (NLC). NLC can be prepared either by blending any solid and liquid lipids or by mixing special combinations of solid and liquid lipids leading to amorphous solids, i.e. hydroxyoctacosanylhydroxystearate and isopropylmyristate (Müller et al., 2002a,b). With regard to these features, high drug payload, avoidance or minimization of drug expulsion and enhancement of chemical stability can be achieved. Therefore, NLC have been represented as a new generation of promising colloidal carriers.

Due to the current ozone layer depletion in the atmosphere, radiation from the sun especially UVA and UVB can reach the earth in a higher amount. Consequently, the radiation can easily expose to skin leading to unwanted and harmful stresses on skin, i.e. skin cancer, wrinkle, dryness and mottled pigment abnormalities. These occur owing to photochemical reaction on the skin as a result of the oxidation reactions. However, the human body has a defense mechanism by producing naturally enzymes and non-enzymatic antioxidants (Hoppe et al., 1999; Kristl et al., 2003; Üner et al., 2005b). Nonetheless, in some situation the antioxidants produced by the body are inadequate. It can be supplemented by oral and/or topical administrations of antioxidants. In the cosmetic field, ascorbic acid has been widely used as an antioxidant for several years. However, due to its stability problem, ascorbyl palmiatate (AP), an ascorbic acid derivative, has been used as an alternative source of ascorbic acid. Its structure is an amphiphilic molecule which is chemically more stable and can penetrate into the skin more easily as compared to its acidic form. However, the chemical stability problem of AP has been demonstrated in many topical preparations (Špiclin et al., 2001, 2003; Kristl et al., 2003; Üner et al., 2005b). In previous reports, sensitive molecules such as retinols (Jenning and Gohla, 2001), ketoconazole (Souto and Müller, 2005) and AP (Üner et al., 2005b) had been successfully incorporated into NLC and the chemical stability was enhanced. Moreover, the enhancement of skin moisturizing of AP-loaded SLN and NLC was higher than that of AP-loaded NE (Üner et al., 2005a). Likewise, sustained release of AP through excised human skin was shown for AP-loaded SLN and NLC in comparison to AP-loaded NE (Üner et al., 2005a). Accordingly, AP-loaded NLC is a promising system in cosmetic use. However, the optimum conditions of AP-loaded NLC have not been reported yet. Therefore, in this study the formulation parameters affecting the stability of AP after incorporation into NLC were evaluated including different types of lipids, types of surfactants, storage conditions, i.e. temperature and nitrogen gas flushing, effects of drug loading as well as types of antioxidants. The investigated parameters in terms of particle size, zeta potential, lipid modification and chemical stability were evaluated.

2. Materials and methods

2.1. Materials

Ascorbyl palmitate (AP), monobasic potassium phosphate, butylate hydroxyanisol (BHA), butylate hydroxytoluene (BHT) and DL-α-tocopherol (Vitamin E) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Imwitor® 900 (glyceryl monostearate) was purchased from Condea (Witten, Germany). Labrafil® M1944 (apricot kernel oil polyethylene glycol-6 ester), Hydrine® (PEG-2 stearate) and Apifil® (nonionic hydrophilic white beeswax) were obtained from Gattefossé GmbH (Cedex, France). Glyceryl monostearate was obtained from Carl Roth (Karlsruhe, Germany). Miranol Ultra® C32 (sodium cocamphoacetate) was purchased from Rhodia (Frankfurt am Main, Germany). Lorol C16® (cetyl alcohol) was obtained from Beierdorf AG (Hamburg, Germany). Tween® 80 (polyoxyethylene sorbitan monooleate) was acquired from Uniqema (Everberg, Belgium). Tego® Care 450 (polyglyceryl-3 methylglucose distearate) was donated from Goldschmidt (Essen, Germany). Ultra purified water was obtained from a MilliQ Plus system, Millipore (Schwalbach, Germany). Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of nanostructured lipid carries (NLC)

The high pressure homogenization technique was applied to produce NLC dispersions according to Müller et al. (2000). Briefly, AP was dissolved in the mixtures of solid and liquid lipids melted approximately 10 °C above the melting point of lipid. Then, the lipid phase was dispersed and admixed to a hot aqueous surfactant solution (80 °C) using an Ultra-Turrax T25 (Janhke & Kunkel GmbH and Co. KG, Staufen, Germany) stirred at 8000 rpm for 1 min. After that the obtained pre-emulsion was subsequently homogenized at 80 °C by a high pressure homogenizer for three cycles at 500 bar using an APV Micron Lab 40 (APV system, Unna, Germany). The hot o/w nanoemulsion was cooled to room temperature leading to the lipid phase recrystallization and finally the NLC were formed. The compositions of formulation ingredients are shown in Table 1.

For the formulation of AP-loaded NLC with antioxidants, the combination of antioxidants was added in the melted lipid phase during the production. Subsequently, the manufacturing process was further performed as mentioned for preparation of AP-loaded NLC.

2.3. Particle size analysis

The mean particle size and polydispersity index (PI) of NLC suspension were measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer IV (Malvern Instruments, UK). PCS yields the mean particle size (z-ave) and polydispersity index as a measure of the width of the particle size distribution. Prior to the particle size measurement, the NLC formulation was diluted with double distilled water.

Table 1
Compositions of the developed NLC formulations (%, w/w)

Compositions	A1	A2	A3	A4	A5	B1	C1	D1	E1	F1	G1	H1
Hydrine®	16.2	16.2	16.2	12.6	12.6	–	–	–	–	16.2	12.6	12.6
Imwitor® 900	–	–	–	–	–	16.2	–	–	–	–	–	–
GS	–	–	–	–	–	–	16.2	–	–	–	–	–
Cetyl alcohol	–	–	–	–	–	–	–	16.2	–	–	–	–
Apifil®	–	–	–	–	–	–	–	–	16.2	–	–	–
Labrafil® M1944	1.8	1.8	1.8	1.4	1.4	1.8	1.8	1.8	1.8	1.8	1.4	1.4
AP	2.0	2.0	2.0	6.0	6.0	2.0	2.0	2.0	2.0	2.0	6.0	6.0
Tego® Care 450	1.8	–	–	1.8	–	–	–	–	–	–	–	–
Tween® 80	–	3.0	–	–	–	–	–	–	–	3.0	3.0	–
Miranol® Ultra® C32	–	–	1.8	–	1.8	1.8	1.8	1.8	1.8	–	–	1.8
BHA	–	–	–	–	–	–	–	–	–	0.02	0.02	0.02
BHT	–	–	–	–	–	–	–	–	–	0.1	0.1	0.1
Vit.E	–	–	–	–	–	–	–	–	–	0.6	0.6	0.6
Water q.s.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

AP, ascorbyl palmitate; GS, glyceryl monostearate; BHA, butylate hydroxyanisol; BHT, butylate hydroxytoluene.

2.4. Polarized light microscopy

The potential presence of drug crystals was monitored by polarized light microscope (Wetzlar, Germany) at 100×, 400× and 1000× magnification equipped with a digital camera. This methodology is a useful tool to select suitable lipids and to detect instability during storage times of NLC owing to drug expulsion.

2.5. Stability studies

All samples were kept in siliconized glass vial at different temperatures (4 °C and 25 °C). To study the effects of nitrogen gas on the stability of the developed AP-loaded NLC formulations, nitrogen was flushed over the top of the formulation to get rid of oxygen. The percentage of AP remaining in NLC formulations was quantified by HPLC at the predetermined time intervals. Briefly, HPLC analysis was performed using a Kroma System 2000 running in the isocratic modus. The system consisted of a HPLC pump 220, an Auto-sampler T360 and a UV detector 430. The stationary phase of HPLC system consisted of 250 mm × 4 mm i.d. column packed with 5 μm Luna-NH_2 (Phenomex, Germany). The mobile phase comprised methanol, acetonitrile and 0.02 M phosphate buffer pH 2.5 (70:10:20). The flow rate was 1 ml/min. The UV detector was set at the wavelength of 254 nm. The injection volume was 20 μl. All samples were preformed in triplicate. The Kontron HPLC software was used for the analysis of the results.

For sample preparation, approximately 250 mg sample was weighed and dissolved in 10 ml of methanol. Then it was sonicated in an ultrasonic bath at 40 °C for 5 min for liberation of AP from lipid nanoparticles. After the suspension was cooled down to room temperature, it was further diluted with mixture of methanol and water (pH adjusted to 2.5 with hydrochloric acid) at the ratio of 9:1 as a diluent.

2.6. Differential scanning calorimetry (DSC)

Thermal analysis was performed using a Mettler DSC 821e apparatus (Mettler Toledo, Gieβen, Switzerland). The samples were weighed for approximately 1–2 mg based on the lipid contents in the formulation in 40 μl aluminum pans. Heating curves were performed from 25 to 85 °C and cooled down to 25 °C at the heating rate of 5 K/min. An empty aluminum pan was used as a reference. The DSC parameters including onset, melting point and enthalpy were evaluated using STAR^e Software (Mettler Toledo, Switzerland). The crystallinity index (CI) was computed using Eq. (1) (Freitas and Müller, 1999).

$$\mathrm{CI}\,(\%) = \left(\frac{\Delta H_{\mathrm{NLC\ aqueous\ dispersion}}}{\Delta H_{\mathrm{bulk\ material}} \times \mathrm{Concentration}_{\mathrm{lipid\ phase}}} \right) \times 100 \quad (1)$$

where ΔH_{NLC} and $\Delta H_{\mathrm{bulk\ material}}$ are the melting enthalpy (J/g) of NLC dispersion and bulk lipid, respectively.

2.7. X-ray diffraction analysis

X-ray diffraction measurement was performed by wide-angle X-ray scattering (WAXS, 2 Theta 4–40°) on a Philips PW1830X-ray generator with a copper anode (Cu Kα radiation, 40 kV, 25 mA, λ = 0.15418), using a Goniometer PW18120 as a detector (Philips, Amedo, the Netherlands). The sample was mounted into a specific device before the measurement by WAXS. The data used were typically collected with a step width of 0.04° and a count time of 60 s. The short spacing diffractograms were calculated using Bragg's equation.

2.8. Statistics

All data were presented as mean ± standard deviation (S.D.). Significance of differences was evaluated using Student's *t*-test and one way ANOVA at the probability level of 0.05.

3. Results and discussion

3.1. Characterization of the developed NLC formulations

Table 1 shows the compositions of the developed NLC formulations. In this study, lipids were selected based on the solubility

Table 2
Mean particle size (*z*-ave), polydispersity index (PI) and zeta potential (ZP) values of the developed NLC formulations after one day storage at room temperature (25 °C)

Formulations	*z*-ave[a]	PI	ZP[b] (mV)
A1	269 ± 7	0.193 ± 0.077	−49.5 ± 0.3
A2	215 ± 33	0.520 ± 0.124	−30.4 ± 2.8
A3	251 ± 6	0.286 ± 0.036	−48.0 ± 1.0
A4	254 ± 5	0.177 ± 0.061	−54.6 ± 0.7
A5	244 ± 4	0.167 ± 0.053	−48.6 ± 0.0
B1	191 ± 3	0.255 ± 0.049	−56.2 ± 1.2
C1	244 ± 5	0.274 ± 0.042	−55.3 ± 0.2
D1	233 ± 3	0.125 ± 0.042	−54.3 ± 0.4
E1	211 ± 3	0.238 ± 0.045	−33.6 ± 1.8

[a] Mean of $n = 10$ measurements.
[b] Mean of $n = 5$ measurements.

screening tests at 80 °C for 1 h and after cooling down to the room temperature of the mixtures of solid lipid, liquid lipid (oils) and AP for 24 h as described by Souto et al. (2005). The presence of drug crystals was detected by polarized light microscopic method. From the results obtained, four solid lipids and one liquid lipid were chosen for preparing lipid nanoparticles based on the NLC systems. In addition, different types of emulsifying agent (surfactant) were also selected to stabilize lipid nanoparticles including Tego® Care 450, Tween® 80 and Miranol Ultra® C32. After production, the mean particle size of all formulations was between 190 and 270 nm and the PI was lower than 0.3 except for the formulation stabilized by Tween® 80 which was approximately 0.5 (Table 2). Concerning the effect of drug loading on the mean particle size, it was found that no correlation was observed. Additionally, after 90 days storage at cold condition (4 °C) and room temperature (25 °C), the mean particle size remained in the nanosize range and less than 350 nm in all formulations. The zeta potential value was revealed to be dependent on the type of surfactants, drug incorporation and lipids (Table 2). NLC stabilized by Tween® 80 showed the lowest value (between −28 and −31 mV); however, this value was around −30 mV indicating good physical stability. Moreover, in case of non-ionic surfactants, the steric hinderance is another additional effect which increases the stability of colloidal dispersions (Lim and Kim, 2002).

In addition, the drug crystal was not observed under polarized light microscope during 90 days storage in all formulations. Therefore, it can be assumed that no drug leakage occurred during the storage time.

3.2. Stability of AP in NLC system

To investigate the formulation parameters affecting the stability of AP-loaded NLC systems, the experiment was carried out over a period of 30 days in case of non-degassing condition whereas that of degassing condition with nitrogen flushing the investigation was prolonged up to 90 days. In this study, the percentage of drug loading was 10% and 30% related to the lipid phase based on the solubility result (data not shown). Due to the high solubility of AP in Hydrine®, AP was incorporated into this lipid at two concentrations as mentioned above whereas in other lipids, 10% of AP related to lipid phase was incorporated into the system. The effects of formulation parameters including the percentage of drug loading, temperature, different types of surfactant and solid lipid, different suppliers and the effect of degassing as well as addition of antioxidants were investigated and compared in terms of the percentage of drug remaining at the predetermined time intervals.

3.2.1. The effects of surfactant type on the stability of AP-loaded NLC

Fig. 1 shows the percentage of AP remaining in the NLC stored in the non-degassing condition which was stabilized using different surfactants including Tego® Care 450 (A1), Tween® 80 (A2) and Miranol Ultra® C32 (A3) at room temperature (25 °C). After 30 days of storage, the highest percentage of AP remaining was AP-loaded NLC stabilized with Miranol Ultra® C32 (46%), followed by Tego® Care 450 (17%) and Tween® 80 (13%). Using one way ANOVA at the significant level (α) of 0.05 to evaluate the data, it was found that the stability of AP-loaded NLC depended significantly on the types of surfactant ($p < 0.05$). Concerning the chemical structure of AP, it composes of cycling ring and hydrocarbon chain (palmitic acid). The cycling ring composes of hydroxyl group which is very sensitive and can be degraded by oxidation reaction. Therefore, enhancement of the stability of AP can be achieved by incorporating the cyclic part into lipid matrix to avoid its exposure to oxygen molecules or by using surfactant which can protect this labile group from oxygen molecule in case of AP located at the interface. However, in this study, the same lipid matrix was selected but stabilized by different surfactants. Generally, it has been recognized that surfactants composing of both polar and non-polar regions adsorb on surfaces or interfaces of the system and modify the surface or interfacial free energy. From the obtained results, it could be stated that the cyclic ring of AP might be located at the interface and the hydrocarbon chain is encapsulated and localized in solid lipid matrix. As a result, types of surfactant are one of the important factors to increase the stability of AP aligned at the interface. A similar finding was also reported in the case of retinol-loaded SLN (Müller et al., 2002b)

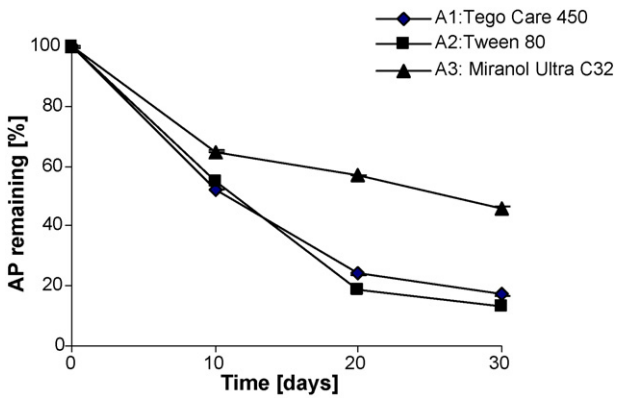

Fig. 1. Percentage of AP remaining at room temperature (25 °C) in non-degassing condition of AP-loaded NLC of Hydrine® stabilized with Tego® Care 450 (A1), Tween® 80 (A2) and Miranol Ultra® C32 (A3).

and AP entrapped in liposomes (Kristl et al., 2003). In case of AP entrapped in liposomes, it was found that chemical stability of AP liposomes composed of non-hydrogenated soybean lecithin (NSL) was higher than that of hydrogenated soybean lecithin (HSL). It was explained that the sensitive molecule of AP could be deeply immersed in the interface when stabilized with NSL compared to that with HSL (Kristl et al., 2003). Besides, it was demonstrated that different types of surfactant showed different effects on the time-course of polymorphic transition after crystallization of the triglyceride nanoparticles of which can be confirmed by X-ray diffraction and thermal analysis (Bunjes et al., 2003).

3.2.2. The effects of degassing on the stability of AP-loaded NLC

The main reason of AP instability is its susceptibility to the oxidation reaction. Therefore, removal of oxygen should practically increase its stability. In this experiment, nitrogen was utilized to replace oxygen on the head space of the formulations A1–A3. Fig. 2 shows the percentage of drug remaining before and after flushing with nitrogen gas. It was found that the percentage of drug remaining after 30 days of storage at room temperature (25 °C) was improved in the degassing condition, i.e. from 17% to 65%, from 13% to 83% and from 46% to 78% in case of A1, A2 and A3, respectively. However, the oxidation reaction still occurred during storage. It might be due to the residual oxygen dissolved in the aqueous phase which was not removed and also the presence of heavy metal during the production process which can catalyze the oxidation reaction. This could be avoided by adding antioxidant and/or chelating agent. Improvement of AP chemical stability after flushing with nitrogen was also observed in particular o/w microemulsions of AP in comparison to w/o microemulsions (Špiclin et al., 2001).

To evaluate the effect of nitrogen gas on the stability of AP-loaded NLC compared with those produced without degassing, Student's *t*-test was used to examine at the significant level (α) of 0.05 at 30 days of storage at room temperature (25 °C). It was shown that the stability of AP-loaded NLC in the degassing condition was better than that in non-degassing condition for all cases ($p < 0.05$). As a result, it can be inferred that stability of AP-loaded NLC could be enhanced by replacing oxygen in the headspace with nitrogen gas.

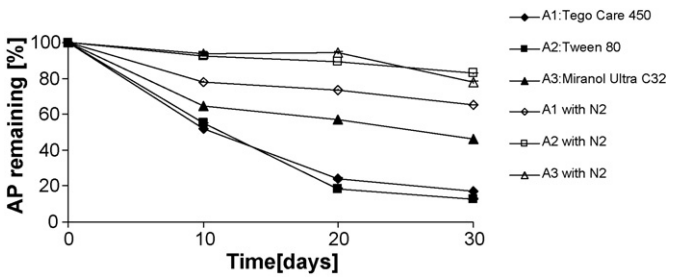

Fig. 2. Percentage of AP remaining observed from the non-degassing condition compared with that from the degassing condition with nitrogen gas of AP-loaded NLC of Hydrine® stabilized with Tego® Care 450 (A1), Tween® 80 (A2) and Miranol Ultra® C32 (A3) stored at room temperature (25 °C).

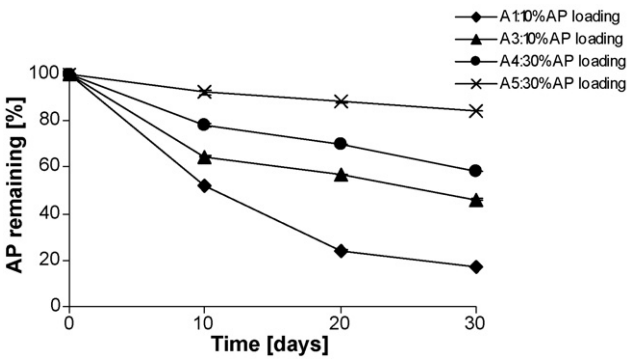

Fig. 3. Percentage of AP remaining at room temperature (25 °C) in non-degassing condition of AP-loaded NLC stabilized with Tego® Care 450 (A1), and Miranol Ultra® C32 (A3) at 10% AP loading (related to lipid phase) in comparison to those (A4 and A5) at 30% AP loading (related to lipid phase).

3.2.3. The effects of drug loading on the stability of AP-loaded NLC

Because of the high solubility of AP in Hydrine®, AP-loaded NLC was developed in two different concentrations as already mentioned above. Comparing formulations A1 and A4 as well as formulations A3 and A5 (10% and 30% AP loading related to lipid phase, respectively), it was observed that the higher AP loading in NLC, the higher chemical stability was obtained. Fig. 3 shows the percentage of drug remaining of the formulations A4 and A5 was 58% and 84%, respectively, after 30 days of storage at room temperature (25 °C). Comparing the mean values of AP remaining of the lower AP loading to that of higher AP loading formulations using Student's *t*-test, significant difference was observed ($p < 0.05$). This might be due to the difference in the drug encapsulation model (Müller et al., 2002a,b). Briefly, during the production by hot HPH technique, drug solubility increases in the water phase leading to the partitioning of drug from the lipid phase to the water phase. However, during cooling of the lipid nanoparticles, drug re-partitioning into the lipid phase occurs. This leads to drug localizing at the interface of nanoparticles. However, increasing of the drug concentration until it reaches the saturation solubility in the melted lipid leads to the 'drug enrich core' model (Mehnert and Mäder, 2001; Müller et al., 2002b). Therefore, AP was likely to be encapsulated in the lipid matrix in case of higher AP loading as compared with that of lower drug loading. As a result, AP molecules could be protected from oxygen molecules and AP chemical stability was enhanced. In a previous report, stability of an AP-like nitroxide probe could be improved after it was incorporated into SLN in comparison to microemulsions (both o/w and w/o) and explained by it being incorporated in the SLN core (Kristl et al., 2003). In addition to the aforementioned reasons, it might be due to the changes in the kinetic order of drug degradation as described in the previously reported case of AP microemulsion (Špiclin et al., 2001).

3.2.4. The effects of solid lipid types on the stability of AP-loaded NLC

Fig. 4 illustrates the percentage of AP remaining in NLC prepared from different types of solid lipid matrices. It was shown

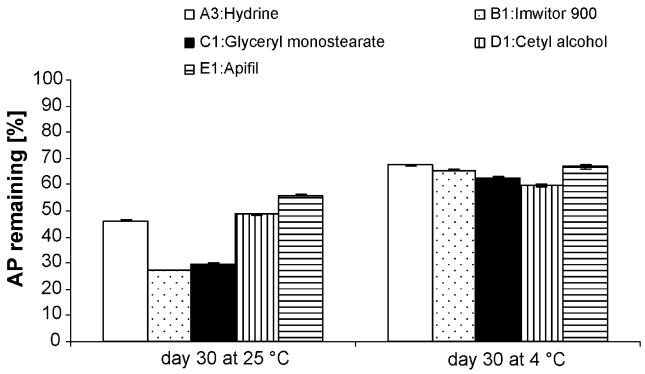

Fig. 4. Percentage of AP remaining in NLC prepared from different types of solid lipid matrices stabilized with Miranol Ultra® C32 at room temperature (25 °C) and cool temperature (4 °C) after 30 days of storage.

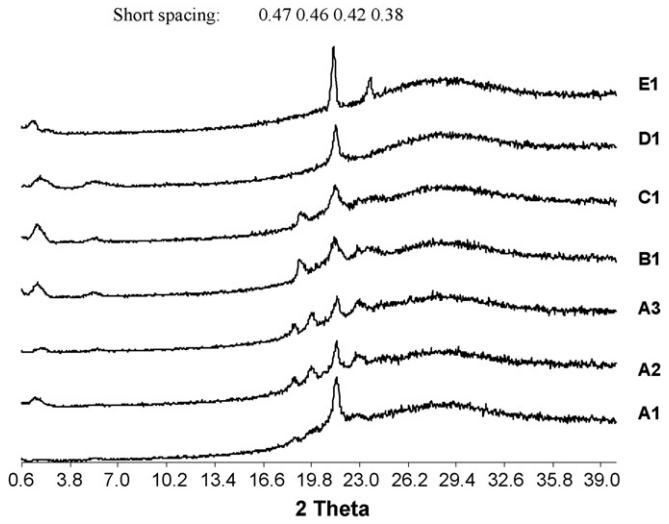

Fig. 5. WAXS diffraction pattern of developed NLC formulations.

that the different types of lipids provided differences in chemical stability. In our experiment, glyceryl monostearate (B1 and C1) showed the lowest percentage of drug remaining after 30 days of storage at room temperature (25 °C). Using one way ANOVA comparing the difference in mean of the percentage of drug remaining, it was found that the chemical stability of AP-loaded NLC using different solid lipid was different from each other ($p < 0.05$). The result leads to the conclusion that the chemical stability of AP was strongly dependent on the type of the solid lipid. In a previous study (Jenning and Gohla, 2001) improvement of retinoids encapsulation and stability was shown after it was incorporated into the NLC. It was concluded that the chemical stability of retinol-loaded NLC prepared from solid lipid having low crystallinity and numerous lattice defects was improved due to enhancement of retinol accommodation inside lipid matrix (Jenning and Gohla, 2000, 2001). Additionally, the chemical structure and the stability of lipid itself should also be considered. For example, encapsulation of all-trans retinol in SLN increased the drug degradation which was explained by the auto-oxidation of lipid inducing the degradation of all-trans retinol (Jee et al., 2006). Therefore, the suitable lipid should be selected during the preformulation process, i.e. evaluation of the stability of lipid and drug in lipid before as well as after incorporation of AP into the NLC. The most suitable lipid might provide the highest chemical stability.

3.2.5. The effects of different lipid suppliers on the stability of AP-loaded NLC

It was previously reported that the properties of SLN dispersion prepared using the same lipid with different batches and/or obtained from different suppliers were varied in terms of its physical stability (Mäder and Mehnert, 2005). Therefore, in this study, glyceryl monostearate (B1 and C1) obtained from different suppliers was selected as a model solid lipid. Their crystallinity patterns were characterized by X-ray diffraction. It was found that glyceryl monostearate from different suppliers showed comparable X-ray pattern both before and after tempering at 80 °C (data not shown). Moreover, X-ray diffraction patterns of both NLC dispersions were similar (Fig. 5). Fig. 4 shows the percentage of AP remaining after 30 days of storage. The obtained results demonstrated that at the day 30 the AP remaining was 27% (B1) and 30% (C1) after storage at room temperature (25 °C) and 65% (B1) and 62% (C1) after storage at cold condition (4 °C). As a result, it can be concluded that in this study glyceryl monosterate obtained from different suppliers provided the same stability for AP-loaded NLC formulations.

3.2.6. The effects of temperature on the stability of AP-loaded NLC

The effects of temperature on drug degradation reaction are generally recognized. It was reported that the reaction rate can be increased with an increase in temperature (Martin, 1993). The effect of temperature on the rate of reaction can be explained by the Arrhenius equation (Martin, 1993). The obtained results revealed that the temperature had a strong effect on the stability of AP-loaded NLC especially in non-degassing condition (compared between 4 °C and 25 °C) (Fig. 4). However, after flushing with nitrogen gas the difference in percentage of AP remaining compared between 4 °C and 25 °C was less than that of the formulations without nitrogen gas flushing (Fig. 6). The main mechanism of AP degradation is oxidation (Špiclin et al., 2001, 2003; Üner et al., 2005b; Kristl et al., 2003). Generally, the oxidation composes of three steps including initiation, propagation and termination. Light and heat are known to initiate the reaction leading to the formation of free radical. This free radical

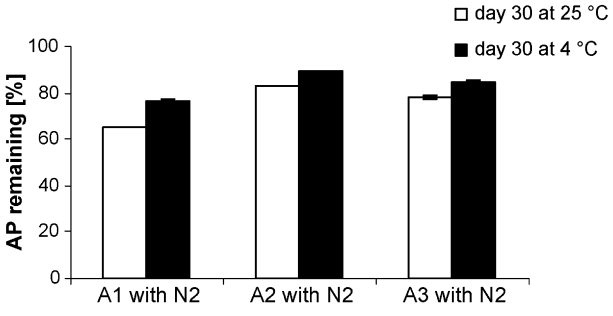

Fig. 6. Percentage of AP remaining in degassing condition at different storage temperature (4 °C and 25 °C) at day 30.

combining with the oxygen molecule causes the propagation step and finally the oxidation progresses to the termination step (chain reactions) (Alexander and David, 2006). Elimination of oxygen and storage at the low temperature (4 °C) can therefore minimize the chain reactions and the oxidation reaction can then be retarded.

Comparing the mean values of AP remaining stored at different temperatures (25 °C and 4 °C) at the day 30 using Student's *t*-test for each formulation, significant difference was shown in all developed formulations ($p < 0.05$) (Figs. 4 and 6).

3.2.7. The effects of antioxidant addition on the stability of AP-loaded NLC

In the preliminary study, the chemical stability of AP in methanol solution could be improved by adding an antioxidant (data not shown). Moreover, it was found that the use of combinations of antioxidants showed a better stability as compared to using individual antioxidant (data not shown). As a result, the combined antioxidants including BHA, BHT and Vitamin E were used to enhance the chemical stability of AP-loaded NLC in this study. Fig. 7 depicts the effects of antioxidants on the chemical stability of AP-loaded NLC in degassing condition. It was shown that after incorporation of antioxidants into the lipid nanoparticles, the chemical stability of AP was improved. To evaluate the effect of antioxidants on the stability of AP-loaded NLC by comparing the formulations with and without addition of antioxidant (F1 and A2) stored at room temperature (25 °C), Student's *t*-test was used to examine at the significant level (α) of 0.05 at the day 30 and 90. The results demonstrated that loading antioxidant into lipid nanoparticles could enhance chemical stability of AP ($p < 0.05$). This was also reported by Jee et al. which chemical stability of all-trans retinol-loaded SLN

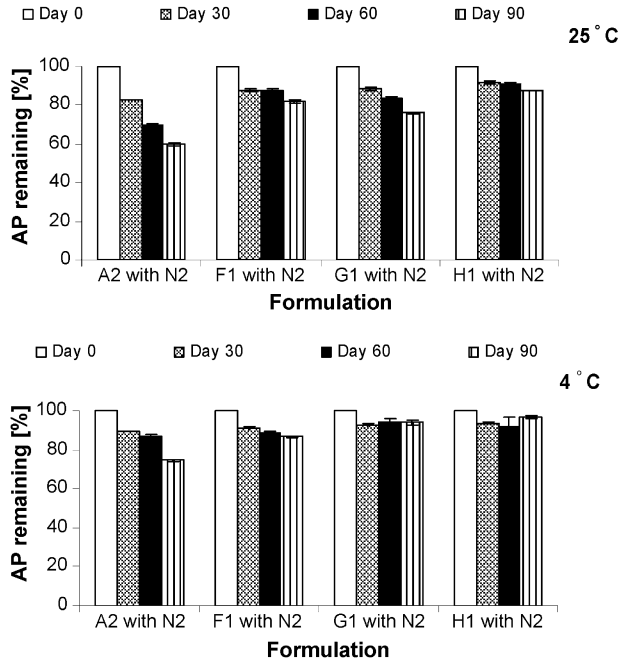

Fig. 7. Effect of antioxidant on the chemical stability of AP-loaded NLC at 25 °C (top) and 4 °C (bottom) at degassing condition.

was improved after incorporation of antioxidants during the production process (Jee et al., 2006). Besides, suitable antioxidant combinations provided the highest chemical stability, i.e. the combination of BHT and BHA in case of all-trans retinol-load SLN. Interestingly, the chemical stability of AP-loaded NLC after a long-term storage (90 days) represented by the percentage of drug remaining was higher than 85% at 4 and 25 °C after flushing the headspace with nitrogen gas and adding the antioxidant [H1].

3.3. X-ray diffraction (XRD) analysis

The molecules composing of long chain compound such as long chain hydrocarbon of fatty acid have been known to possess polymorphism (Sato, 2001). In this study, several types of solid lipid were selected to prepare lipid nanoparticles. The crystalline order of the lipid nanoparticles was elucidated by wide-angle X-ray diffraction. Fig. 5 shows the scattering pattern of all developed formulations. It was clearly shown that no peak of AP was detected in all diffractograms. This confirms that AP was molecularly dispersed (dissolved) in the lipid phase of NLC. Table 3 presents XRD short spacing of polymorphs of AP-loaded NLC formulations. It was found that the AP-loaded NLC containing glyceryl monostearate showed β'/β_i modification, the AP-loaded NLC containing cetyl alcohol showed α modification and the AP-loaded NLC containing Apifil® showed orthorhombic subcell (β'-form). Regarding the X-ray diffractogram of Hydrine®, it was shown that the X-ray patterns obtained depended on the stabilizers. It was demonstrated that Hydrine® stabilized by Tego® Care 450 indicated α modification whereas that stabilized by Tween® 80 and Miranol Ultra® C32 demonstrated the combination of several polymorphic modifications. It has been known that triacylglycerides (TAG) have three polymorphs including α, β' and β forms (Sato, 2001; Bunjes et al., 1996). The α-form shows disordered inner structure while the β-form has the densest packed inner structure. Consequently, they possess different properties in terms of crystallization rate, crystal size and crystallinity. In addition, external factors, for instance, temperature, pressure, impurity, etc. strongly influence the crystal structure. Therefore, the lipid nanoparticles may have a different polymorphism compared with the bulk material (Sato, 2001). Moreover, the influence of surfactant on the crystal behavior of lipid has been previously reported (Bunjes et al., 2003). To summarize, it can be deduced that the polymorphism of lipid had influences on the stability of AP. Comparing the NLC stabilized by the same surfactant but prepared by differ-

Table 3
XRD short spacing values of developed NLC formulations

Formulations	*d*-Spacing (nm)	Polymorph
A1	0.41	α
A2	0.38, 0.41, 0.46, 0.47	$\beta' + \beta$
A3	0.38, 0.41, 0.46, 0.47	$\beta' + \beta$
B1	0.38, 0.41, 0.46	β'/β_i
C1	0.38, 0.41, 0.46	β'/β_I
D1	0.41	α
E1	0.38, 0.41	β'

Table 4
DSC parameters of AP-loaded NLC formulations

Formulations	Onset (°C)	Peak maximum (°C)	Enthalpy (J/g)	CI (%)
A1	45.05	49.73	120.45	82.89
A2	47.38	48.27	127.15	87.50
A3	43.55	48.70	123.85	85.23
A4	49.98	52.51	88.90	61.18
A5	47.50	51.08	97.15	66.85
B1	55.04	57.80	121.20	70.02
C1	53.69	56.49	115.10	68.26
D1	50.49	56.85	98.50	42.01
E1	55.74	60.42	108.70	85.42

ent solid lipid matrices at room temperature, it was found that AP-loaded NLC containing glyceryl monostearate showed the lowest chemical stability (Fig. 4). Concerning the X-ray pattern of AP-loaded NLC prepared with glyceryl monostearate, it possessed the broad reflections, compared to the sharp reflections of that prepared with Apifil® indicating the high crystal order of the inner structure of the lipid particles. Therefore, in this case AP entrapped in glyceryl monostearate NLC was likely to be located in the inner structure of the nanoparticles. Hence, theoretically, the stability of AP entrapped in glyceryl monostearate NLC could be enhanced compared with that in Apifil® NLC as described for retinol-loaded NLC (Jenning and Gohla, 2000, 2001). However, only this factor alone is not sufficient to explain the obtained result because after storage for 30 days at room temperature (25 °C), AP-loaded NLC prepared with glyceryl monostearate showed the lowest AP remaining. In contrast, the chemical stability of AP-loaded NLC after storage for 30 days at 4 °C was closed to each other as compared with that stored at 25 °C (Fig. 4). This might be due to the retardation of AP degradation rate and lipid auto-oxidation which occurred at the low temperature storage as already mentioned above. As a result, the chemical stability of lipid itself can be included as another factor to enhance the chemical stability of AP-loaded NLC.

3.4. DSC analysis

Table 4 shows the DSC parameters including melting point, onset, enthalpy and crystallinity index (CI) after 3 days of storage at room temperature (25 °C). A super-cooled melt was excluded due to the presence of melting enthalpy in all formulations. Moreover, all formulations showed onset and melting point of higher than 40 °C which is the prerequisite when lipid nanoparticles are applied for skin delivery (Saupe et al., 2005). Comparison of 10% and 30% AP loading related to lipid content (A1 and A4, A3 and A5), it was found that higher drug loading decreased the crystallinity of the lipid nanoparticles. After storage for 90 days, the crystallinity increased in all formulations and was more pronounced when they were stored at room temperature (data not shown). Generally, the increase of the CI (%) during storage leads to the instability of lipid nanoparticles due to the solidification of the liquid suspension as previously reported by Freitas and Müller (1999). It was explained that an increase in viscosity of the suspension was accompanied by the lack of lattice defects within lipid particles (Freitas and Müller, 1999). As a result, the drug loading was reduced. This is another reason why the chemical stability of AP-loaded NLC stored at 4 °C was higher than that stored at 25 °C. The increasing of CI (%) was stimulated, for example, by high storage temperature, by exposure to light and by shear force input to the lipid nanoparticles. The physical stability of SLN dispersions after storage in suitable conditions (8 °C) for 3 years was reported (Freitas and Müller, 1999).

4. Conclusions

The chemical stability of AP-loaded NLC can be improved by selecting suitable types of lipid, surfactant, and proper storage conditions, i.e. cold temperature and flushing with nitrogen gas or inert gas. Moreover, the instability of AP was overcome by incorporation of antioxidants into NLC during the production step and the percentage of AP-loaded NLC remaining after storage for 90 days still was more than 90% after flushing with nitrogen gas, adding combined antioxidants and storage at 4 °C.

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References

Alexander, T.F., David, A., 2006. Drug stability. In: Alexander, T.F., David, A. (Eds.), Physicochemical Principles of Pharmacy. Pharmaceutical Press, London, pp. 93–138.

Bunjes, H., Koch, M.H., Westesen, K., 2003. Influence of emulsifiers on the crystallization of solid lipid nanoparticles. J. Pharm. Sci. 92, 1509–1520.

Bunjes, H., Westesen, K., Michel, H.J.K., 1996. Crystallization tendency and polymorphic transitions in triglyceride nanoparticles. Int. J. Pharm. 129, 159–173.

Cavalli, R., Gasco, M.R., Chetoni, P., Burgalassi, S., Saettone, M.F., 2002. Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin. Int. J. Pharm. 238, 241–245.

Dingler, A., Krohs, S., Lukowski, G., Gohla, S., Müller, R.H., 1996. SLN (Solid lipid nanoparticles) as new solid carrier of active ingredients in cosmetics. Eur. J. Pharm. Sci. 4, S148–S1148.

Freitas, C., Müller, R.H., 1999. Correlation between long-term stability of solid lipid nanoparticles (SLN(TM)) and crystallinity of the lipid phase. Eur. J. Pharm. Biopharm. 47, 125–132.

Hoppe, U., Bergemann, J., Diembeck, W., Ennen, J., Gohla, S., Harris, I., Jacob, J., Kielholz, J., Mei, W., Pollet, D., Schachtschabel, D., Sauermann, G., Schreiner, V., Stab, F., Steckel, F., 1999. Coenzyme Q10, a cutaneous antioxidant and energizer. Biofactors 9, 371–378.

Jee, J.P., Lim, S.J., Park, J.S., Kim, C.K., 2006. Stabilization of all-trans retinol by loading lipophilic antioxidants in solid lipid nanoparticles. Eur. J. Pharm. Biopharm. 63, 134–139.

Jenning, V., Gohla, S., 2000. Comparison of wax and glyceride solid lipid nanoparticles (SLN®). Int. J. Pharm. 196, 219–222.

Jenning, V., Gohla, S.H., 2001. Encapsulation of retinoids in solid lipid nanoparticles (SLN). J. Microencapsul. 18, 149–158.

Kristl, J., Volk, B., Gasperlin, M., Šentjurc, M., Jurkovič, P., 2003. Effect of colloidal carriers on ascorbyl palmitate stability. Eur. J. Pharm. Sci. 19, 181–189.

Lim, S.J., Kim, C.K., 2002. Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. Int. J. Pharm. 243, 135–146.

Martin, A., 1993. Kinetics. In: Martin, A., Bustamente, P., Chou, A.H.C. (Eds.), Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences, London, pp. 284–323.

Mäder, K., Mehnert, W., 2005. Solid lipid nanoparticles—concepts, procedures, and physicochemical. In: Claudio, N. (Ed.), Liposphers in Drug Targets and Delivery: Approaches, Methods, and Applications. CRC Press, New York, pp. 2–18.

Mehnert, W., Mäder, K., 2001. Solid lipid nanoparticles: production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165–196.

Müller, R.H., Maassen, S., Schwarz, C., Mehnert, W., 1997. Solid lipid nanoparticles (SLN) as potential carrier for human use: interaction with human granulocytes. J. Controlled Release 47, 261–269.

Müller, R.H., Mäder, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.

Müller, R.H., Radtke, M., Wissing, S.A., 2002a. Nanostructured lipid matrices for improved microencapsulation of drugs. Int. J. Pharm. 242, 121–128.

Müller, R.H., Radtke, M., Wissing, S.A., 2002b. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv. Drug Deliv. Rev. 54, S131–S155.

Pandey, R., Sharma, S., Khuller, G.K., 2005. Oral solid lipid nanoparticle-based antitubercular chemotherapy. Tuberculosis 85, 415–420.

Sato, K., 2001. Crystallization behaviour of fats and lipids—a review. Chem. Eng. Sci. 5, 2255–2265.

Saupe, A., Wissing, S., Lenk, A., Schmidt, C., Müller, R.H., 2005. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)—structural investigations on two different carrier systems. Biomed. Mater. Eng. 15, 393–402.

Schwarz, C., Mehnert, W., 1997. Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). Int. J. Pharm. 157, 171–179.

Sivaramakrishnan, R., Nakamura, C., Mehnert, W., Korting, H.C., Kramer, K.D., Schäfer-Korting, M., 2004. Glucocorticoid entrapment into lipid carriers—characterisation by parelectric spectroscopy and influence on dermal uptake. J. Controlled Release 97, 493–502.

Souto, E.B., Anselmi, C., Centini, M., Muller, R.H., 2005. Preparation and characterization of n-dodecyl-ferulate-loaded solid lipid nanoparticles (SLN®). Int. J. Pharm. 295, 261–268.

Souto, E.B., Müller, R.H., 2005. SLN and NLC for topical delivery of ketoconazole. J. Microencapsul. 22, 501–510.

Špiclin, P., Gašperlin, M., Kmetec, V., 2001. Stability of ascorbyl palmitate in topical microemulsions. Int. J. Pharm. 222, 271–279.

Špiclin, P., Homar, M., Zupancic-Valant, A., Gašperlin, M., 2003. Sodium ascorbyl phosphate in topical microemulsions. Int. J. Pharm. 256, 65–73.

Üner, M., Wissing, S.A., Yener, G., Müller, R.H., 2005a. Skin moisturizing effect and skin penetration of ascorbyl palmitate entrapped in solid lipid nanoparticles (SLN) and nanostructured lipid carries (NLC) incorporated into hydrogel. Pharmazie 60, 751–755.

Üner, M., Wissing, S.A., Yener, G., Müller, R.H., 2005b. Solid lipid nanoparticles (SLN) and nanostrutured lipid carriers (NLC) for application of ascorbyl palmitate. Pharmazie 60, 577–582.