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ORIGINAL ARTICLE

Production and characterization of testosterone undecanoate-loaded NLC for oral bioavailability enhancement

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Abstract

Purpose: Nanostructured lipid carriers were loaded with testosterone undecanoate (TU), which has a low oral bioavailability. *Methods*: Different NLC dispersions were produced using the hot high pressure homogenization method. Particles were characterized using dynamic and static light scattering techniques, differential scanning calorimetry and X-ray diffraction. And the bioavailability was compared to a marketed product. *Results*: Nanostructured lipid carriers with up to 30% TU load and sizes of about 600 and 200 nm could be achieved, allowing a direct comparison of the size effect in in vivo bioavailability studies. The zeta potentials varied between −20 and −40 mV. The bioavailability of Andriol Testocaps® in the fed state was matched. *Conclusions*: This opens the perspective of administering a single dose of dose of TU in one oral dosage unit and simultaneously having a bioavailability less dependent on the fed state.

Key words: DSC, lipid nanoparticles, NLC, oral bioavailability, testosterone undecanoate

Introduction

The bioavailability of testosterone is relatively low (~1%) because of the pronounced first-pass metabolism of the liver[1–3]. Lipophilic derivatives of testosterone [e.g., testosterone undecanoate (TU)] possess a higher bioavailability of about 3% in beagle dogs and 7% in man[4,5]. Therefore, TU is used in commercial preparations, for example, Andriol® and Andriol Testocaps®[5]. The latter one is a solution of TU in castor oil and Lauroglycol FCC. Because of the relatively low solubility of TU, a single dose of 80 mg needs to be administered in two capsules. The weight of the total solution in these two capsules is 770 mg. Obviously, two capsules are not patient-friendly and multiple units reduce the compliance. Moreover, the bioavailability of Andriol Testocaps® is strongly dependent on the nonfed/fed status of the patient[6,7].

Therefore, there is a definite need for an improved oral TU formulation. Nanoparticulate delivery systems are known to increase the oral bioavailability[8–10]. In particular, lipids as an excipient are also promoting the oral absorption[5,11,12]. The nanostructured lipid carriers (NLC) unify in one delivery system both aspects being nanoparticulate and composed of lipids as particle matrix. Oral bioavailability enhancement could be shown, for example, for cyclosporine[13,14] and for the drugs fenofibrate[8], naproxen[9], and danazol[10]. The advantage of the nanoparticles is that they also show a lymphatic uptake when their size is below about 100 nm, the cutting-edge size for lymphatic absorption[10]. The TU bioavailability in the blood is mainly a result of lymphatic absorption. Therefore, a nanoparticulate delivery system could exploit the lymphatic route for leading to an improved bioavailability.

In this study, solid lipids and oils were screened regarding their solubility properties for TU to be used in NLC production. NLC were produced with different matrix compositions and sizes to allow the study of these effects on the in vivo bioavailability. The particles were characterized regarding size, surface charge, structure of

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matrix, and short-term stability with the perspective of developing an improved oral formulation.

Materials and methods

Materials

TU was bought from Jenapharm (Jena, Germany). Testosterone, oleic acid, and castor oil were obtained from Cealo (Casar & Lorete GmbH, Hilden, Germany), Dynasan 118 from Condea (Germany), stearic acid from Sigma-Aldrich (Munich, Germany), and Carnauba wax from Roth (Karlsruhe, Germany). Tween 80 (polysorbate 80, Sigma-Aldrich, Germany) was used as stabilizer for all the formulations investigated. Purified water was obtained from a MilliQ system (Millipore, Germany). All other chemicals used were of analytical grade.

Methods

Production of NLC

The NLC were produced by melting the lipid blend at 75°C, dissolving the TU (achieved from PharmaSol GmbH, Berlin, Germany), and homogenizing the TU-containing blend in a hot Tween 80 solution of identical temperature. The Tween 80 concentrations used were 1% and 2%, respectively. The obtained coarse macro-emulsion was homogenized using a Micron Avestin Emulsiflex B3 homogenizer (Avestin Europe, Mannheim, Germany). The applied production pressure and the number of cycles were varied to modify the particle size: 200–500 bars and 1–3 cycles. For homogenization at 75°C, the Avestin Emulsiflex B3 was equipped with a temperature control jacket.

Particle size analysis

Particle size was analyzed by photon correlation spectroscopy (PCS) using a Malvern ZetaSizer 2000 HS (Malvern Instruments, Malvern, UK). The measuring mode applied was the Contin-Auto mode. PCS yields the mean diameter of the bulk population (z-average) and a polydispersity index (PI) ranging from 0 (monodisperse) through 0.10–0.20 (relatively monodisperse) to >0.5 for a broad size distribution. The measuring range of PCS is approximately 3 nm–3 μm.

For detection of aggregates in the micrometer range formed during the storage of the NLC suspensions, laser diffractometry (LD) was employed. A Malvern Mastersizer X (Malvern Instruments) was used to apply the Mie theory, real refractive index of 1.465, and imaginary refractive index of 0.01. LD yields a volume distribution, in contrast to PCS yielding an intensity-weighted diameter. LD diameters selected for characterizing the NLC dispersions were 50% and 90%.

Differential scanning calorimetry

The particle matrix structure was investigated by differential scanning calorimetry (DSC) using a Mettler Toledo DSC821e (Mettler-Toledo, Giessen, Germany). For analysis, the materials were weighted in 40 mg pans. Heating and cooling rates were 5 K/min, respectively. The thermograms were analyzed using the STAR software.

X-ray diffraction patterns were measured using a Philips X-ray generator PW 1830 equipped with a copper cathode (λ = 1.5418Å, 40 kV, 20 mA). The scattered radiation was measured with a vertical goniometer (Philips PW 1820, Philips Industrial & Electro-Acoustic Systems Division, Almelo, The Netherlands). Analysis was performed with computer-interfaced Philips PW 1710 diffractometer control unit.

Zeta potential measurements

The zeta potentials (ZPs) were also determined by the Malvern ZetaSizer (Malvern Instruments). The applied field strength was about 20 V/cm and the electrophoretic mobility was converted to the ZP by applying the Helmholtz–Smoluchowski equation[15]. Measurements were performed in distilled water with the conductivity adjusted to 50 μS/cm using sodium chloride solution. Adjustment to 50 μS/cm avoids fluctuations of the ZPs because of changes in the quality of distilled water (i.e., the conductivity which can fluctuate between 1 and 10 μS/cm)[16].

Light microscopy

The NLC dispersions were also characterized by light microscopy using a microscope Leitz Orthoplan (Leitz, Wetzlar, Germany). Analysis was performed applying a 400× magnification and oil immersion. The intention to use light microscopy was to screen for particles and aggregates larger than 1 μm. To increase the probability of detecting such particles, the NLC suspensions were analyzed undiluted.

Results and discussion

Lipid screening and testosterone undecanoate solubility

Two capsules of Andriol Testocaps® are required to deliver one single dose because the solubility of TU is limited in the capsule oil mixture (~10%, 80 mg TU in 770 mg oil solution in two capsules). To load the NLC with a dose as high as possible, a lipid screening was performed determining the solubility of TU in oils and in melted solid lipids. The primary focus was on the lipids that appeared promising for delivery of TU according to theoretical considerations. According to Charman, glycerides with fatty acids with a carbon chain length of 16–18 atoms have the greatest potential to increase lymphatic absorption[11,17,18]. Therefore, Dynasan 118 was chosen as triglyceride of stearic acid (which is a C_{18} fatty acid). To look at the effect of the lipid structure, triglyceride versus fatty acid, stearic acid itself was chosen as another C_{18} lipid. Admixing of oil to a solid lipid reduces the melting point. As a result, to achieve a high oil load, it is favorable

Table 1. Solubility (%) of testosterone undecanoate in oils at room temperature and in oils, molten lipids, and blends of oils and molten lipids at 75°C after 30 minutes.

Lipid	Solubility (25°C)
Oleic acid	25%
Castor oil	20%
	Solubility (75°C)
Oleic acid	30%
Castor oil	25%
Dynasan 118	30%
Stearic acid	25%
Carnauba wax	30%
Oleic acid/Dynasan 118 (1 : 1)	35%
Oleic acid/stearic acid (1 : 1)	30%
Carnauba wax/oleic acid (1 : 1)	40%

to have lipids with a very high melting point. Carnauba wax with a melting point of 82–86°C was chosen because of previous experiences. It allowed incorporation of up to 60% oil and yielded NLC still solid at 40°C. Oleic acid was included as oil component because it is contained in Andriol® and possesses very good solubility properties for TU. As an alternative, castor oil was also investigated. It is the oil component of Andriol Testocaps®. The screening was done in steps of 5%.

Table 1 shows the different solubilities. Oleic acid was superior in dissolution of TU compared to castor oil having a 5% higher solubility both at room temperature and at 75°C. The solubility of TU in the melted stearic acid was 25% and for Dynasan 118 and carnauba wax, it was 30%. Dissolving TU in the mixtures of oil and lipid increased the solubility in all mixtures by 5% compared to the best solubility in one of the single components. This can be explained by the difference in dissolution behavior between ideal binary mixtures of two solvents and real mixtures. In ideal binary mixtures, the solubility in a mixture is the sum of the solubility in both components, based on the mole fractions of each of the components. In real binary mixtures there can be a negative or positive deviation yielding lower or higher solubility. Fortunately, in the investigated real mixtures the solubility of TU was higher, which was favorable for the production of NLC.

In general, the solubility of a component in a solid lipid is lower than in a liquid lipid. Therefore, admixing an oil to the solid lipid to produce NLC leads to an increased drug loading, when compared to the first generation of lipid nanoparticles, the solid lipid nanoparticles[19,20]. In addition, the presence of the oil molecules distorts the formation of perfect lipid crystals of the solid lipid. This leads to imperfections, which is favorable for drug inclusion[21].

Production of NLC: size

For the production of NLC, mixtures of Dynasan 118 with oleic acid (50 : 50) were chosen. The basic drug loading was 15%. The rationale behind was to have a high lipid to drug ratio (85 : 15) to exploit the absorption promoting properties of the lipid. Two different concentrations of Tween 80 were chosen (1% and 2%) to obtain particles with different sizes. This should allow investigating the size effect but maintaining the same composition of the particle matrix and drug loading. One formulation was produced with 30% drug content to investigate in vivo the effect of a reduced lipid to drug ratio (70 : 30). Can a similarly high bioavailability still be achieved at the lower ratio? The fourth formulation was chosen to compare the effect of glyceride versus fatty acid in the particle matrix, that is, producing NLC with stearic acid/oleic acid (50 : 50) instead of using Dynasan 118. For these particles, again 2% Tween 80 was chosen, because from theoretical considerations small particles should result in a higher bioavailability compared to larger NLC. Table 2 gives an overview of these four NLC formulations.

Table 3 shows the mean PCS diameters and the PIs of these four formulations. As expected, the formulation with only 1% Tween 80 and homogenized with just one homogenization cycle and 200 bars yielded a relatively large size of about 600 nm. Increasing the concentration to 2% Tween 80 and applying three homogenization cycles and 500 bars yielded a size of about 200 nm for both the Dynasan 118/oleic acid NLC loaded with 15% and 30%, respectively. This allowed comparing in vivo large-size versus small-size NLC (identical lipid : drug ratio). Furthermore, small-size NLC were compared with high and low lipid to drug ratio 85 : 15 and 70 : 30 (i.e., a lower and higher drug loading, 15% and 30% TU). In formulation 4, Dynasan 118 was replaced by stearic acid (15% TU) and also a size of about 200 nm was achieved. Therefore, all target sizes could be produced for the envisaged in vivo study. The PI for all formulations was around 0.20 indicating a relatively narrow size distribution. This was confirmed by the LD diameters 50% and 90%. For example, the diameter 90% is 0.91 μm for the larger sized NLC1 and about 0.50 μm for the other three NLC formulations 2, 3, and 4 (Table 4). The LD

Table 2. Overview of the formulations used: all formulations contained a 10% lipid phase (=solid lipid, oil, and drug).

Formulation	Tween 80 (%)	Lipids (solid lipid/liquid lipid)	TU content of lipid phase (%)
NLC1	1	Dynasan 118/oleic acid (50/50)	15
NLC2	2	Dynasan 118/oleic acid (50/50)	15
NLC3	2	Dynasan 118/oleic acid (50/50)	30
NLC4	2	Stearic acid/oleic acid (50/50)	15

Table 3. PCS sizes of the formulations directly after production ($n = 3$).

Formulation	PCS size (nm)	PI
NLC1	592 (±7)	0.15 (±0.02)
NLC2	176 (±0)	0.23 (±0.01)
NLC3	215 (±3)	0.15 (±0.01)
NLC4	200 (±3)	0.23 (±0.02)

Table 4. Laser diffractometer measurements of the formulations directly after production.

Formulation	LD50% (μm)	LD90% (μm)
NLC1	0.37	0.91
NLC2	0.25	0.40
NLC3	0.28	0.48
NLC4	0.29	0.54

diameters 50% are in general higher than the PCS diameters because LD yields a volume size distribution. The diameters 50% confirm also that NLC1 are larger than the other NLC formulations and NLC2 to NLC4 are similar in the diameter 50% (0.25–029 μm).

Structural investigations

The bulk material of TU and the blends of the solid lipids with the oils were investigated. Figure 1 shows exemplarily the DSC curve of TU and the blend of Dynasan 118 and oleic acid. The TU melts at 64.4°C and the peak maximum of the lipid blend is at 66.6°C. Figure 2 shows exemplarily the DSC curves after incorporation of 15%

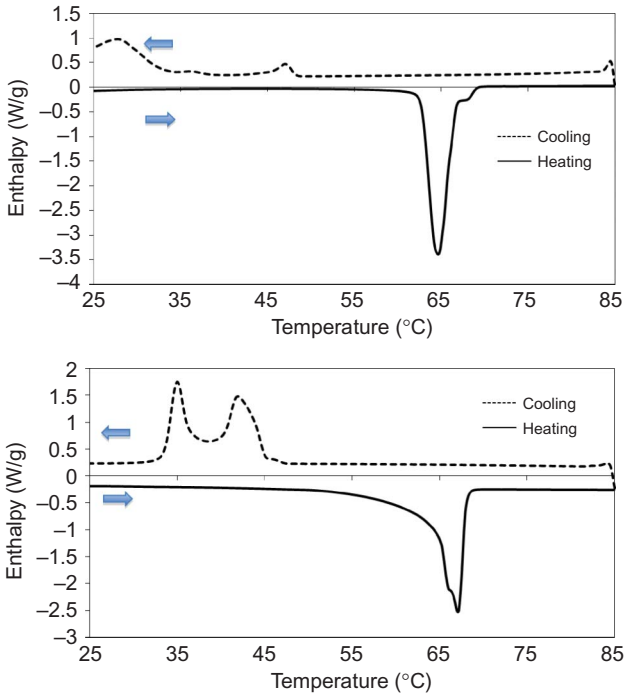

Figure 1. DSC curve of the bulk material of testosterone undecanoate (upper) and a blend of Dynasan 118 and oleic acid (50/50) (m/m) (lower).

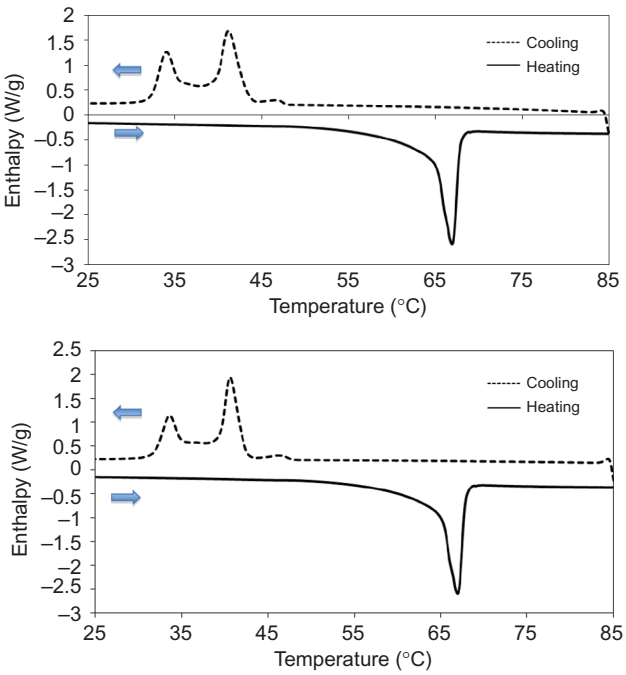

Figure 2. DSC curve of the bulk material of a blend of 15% testosterone undecanoate, 42.5% Dynasan 118, and 42.5% oleic acid (upper) and a blend of 30% testosterone undecanoate, 35% Dynasan 118, and 35% oleic acid indicating the complete solution of TU in the lipids.

and 30% TU in these matrices. The melting peaks are at 66.5°C and 65.4°C showing only a slide reduction in the melting point because of TU. From the distinct sharp shape of the melting peaks, it can be concluded that TU is molecularly dispersed in the blend and not precipitated as a separate crystalline fraction. The cooling curves of the DSC show a recrystallization of TU in two peaks, a minor peak at 47.0°C and a major peak at 27.9°C (Figure 1, upper). In contrast to this, a bimodal peak is obtained with the lipid blend having peak maxima at 42.2°C and 35.3°C (Figure 1, lower). The shape of this recrystallization peak remains practically the same, apart from an increase of the peak height at about 42°C when moving from 15% to 30% TU (Figure 2). A separate recrystallization peak of TU is absent, supporting also the assumption that TU is molecularly dispersed. At least no crystalline TU is present.

This is confirmed by the X-ray diffractograms. Figure 3 shows the X-ray diffraction patterns of the bulk material of TU and the blend of Dynasan 118 and oleic acid. Analysis of the NLC dispersions revealed an amorphous halo because of the presence of water, superimposed with the peaks of the lipid, the TU peaks were absent. Figure 4 shows this exemplarily for 15% and 30% TU incorporated in a matrix of Dynasan 118/oleic acid. Crystalline TU is absent or, to be more precise, below 5%, which is the detection limit of the applied X-ray diffraction.

The NLC suspensions were also analyzed by light microscopy using 400-fold magnification (Figure 5). The

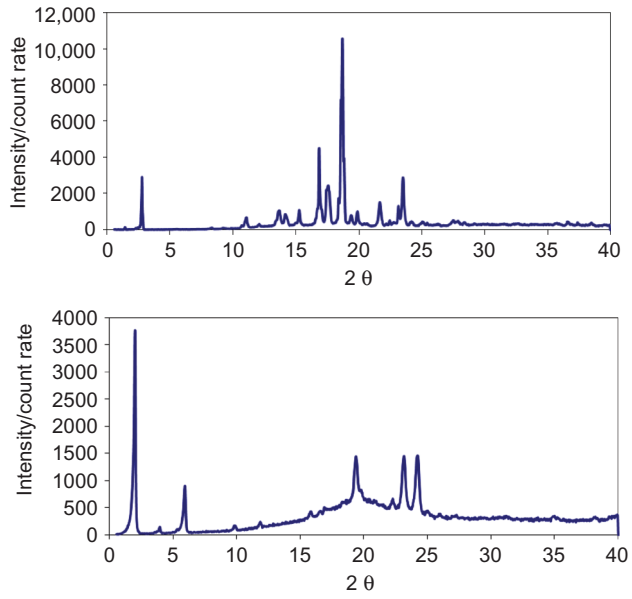

Figure 3. X-ray diffraction pattern of the bulk material of TU (upper) and a blend of oleic acid and Dynasan 118 (50/50) (m/m) (lower).

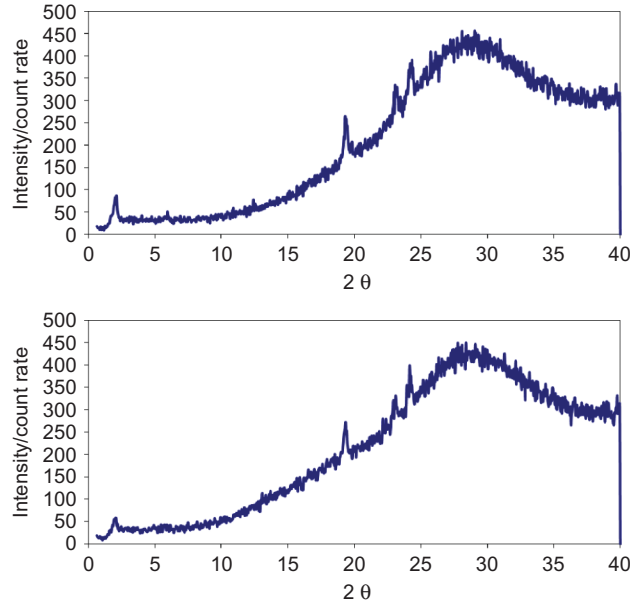

Figure 4. X-ray diffraction pattern of a 10% aqueous NLC dispersion made of 15% TU (upper) and 30% TU (lower) in Dynasan 118 and oleic acid (50/50) showing a solution of TU in both concentrations. None of the characteristic peaks of the TU bulk material can be found in the patterns.

pictures taken directly after production show a more coarse suspension for the formulation NLC1 with a mean PCS diameter of 600 nm. The formulations NLC2 and NLC3 show a very fine appearance. With a mean PCS diameter of about 200 nm the main population is below the detection limit of the microscope. The larger particles in these two formulations detectable with the microscope reveal that they are relatively small. Formulation NLC4 shows a slighter coarse image, which is in agreement with the tendency to aggregate, found in the short-term stability study (see below).

Zeta potential

The ZP is a parameter to roughly estimate the physical stability of dispersions. The ZP measured in distilled water is set to be identical to the Stern potential. The Stern potential is a function of the Nernst potential of the surface. The higher the Nernst potential, the higher the Stern potential. Therefore, in theory, the higher is the ZP, the higher will be the physical stability of the system.

The ZPs measured in distilled water were lowest for NLC1 (−18.6 mV), about −30 mV for NLC2 and NLC3, and about −40 mV for NLC4 (Table 5). The high ZP for NLC4 can be explained by the presence of stearic acid introducing additional charges to the particle surface, leading to a higher Nernst potential. Based on these data, least stability was expected for formulation NLC1, but highest for formulation NLC4.

Short-term stability

The NLC suspensions were stored at room temperature and the size monitored as a function of storage time over 3 months. Table 6 shows the PCS and LD data. NLC1 were not stable, this is in agreement with the measured lower ZP. After 1 week, the suspensions showed microscopically visible large aggregates and size measurements were not continued. The NLC2 and NLC3 were stable over a period of 3 months. There was little change in PCS mean sizes and LD diameters. Surprisingly, NLC4 were found to be also unstable at measuring point 1 week. Because of the highest ZP measured, a better stability was expected. However, these instabilities can be explained by bridging phenomena that some fatty acids and longer chain alcohols can cause[22]. Nevertheless, it should be pointed out that for a pharmaceutical oral formulation a stability of just 1 day is fully sufficient because the aqueous suspensions will be transferred to a tablet or a spray-dried product anyway. That means immediately after NLC production the subsequent processing step to the final dosage form can be performed.

In vivo bioavailability

The bioavailability of the produced NLC dispersions was investigated in rats[23]. The NLC suspensions were administered through a gavage, the dose was 10 mg TU per kg body weight. As a comparison, Andriol Testocaps® were investigated. To simulate the nonfed state, the Andriol Testocaps® solution was dispersed in aqueous Tween 80 solution. Dispersion was necessary to obtain a minimum volume, which could be administered to the rats. To simulate the fed status, the Andriol Testocaps® solution was diluted with castor oil/Lauroglycol FCC solution present in the capsules. All NLC formulations exhibited an area under the curve (AUC) between about 10,000 and 14,000 pg·h/mL, whereas Andriol Testocaps® in

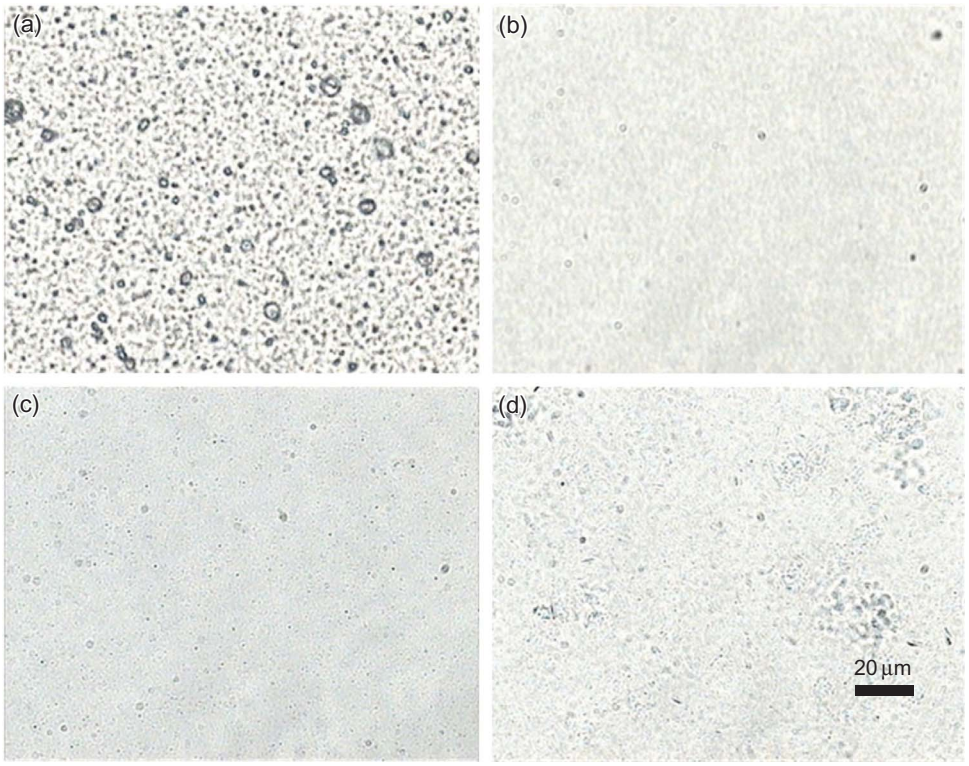

Figure 5. Light microscopic pictures of the NLC formulations: (a) NLC1, (b) NLC2, (c) NLC3, and (d) NLC4. The formulation with the larger particles (NLC1) can be clearly distinguished from NLC2 and NLC3 (which have similar compositions). NLC4 have a different pattern because of a different solid lipid used (stearic acid).

Table 5. Zeta potential results: measurements were performed in ultrapure water adjusted to a conductivity of 50 µS.

Formulation	Zeta potential (mV)
NLC1	−18.6
NLC2	−29.3
NLC3	−29.8
NLC4	−38.5

Tween solution exhibited only a bioavailability of about 8500 units (Table 7).

The increase in bioavailability from formulation NLC1 to NLC2 and NLC3 can be explained by the decrease in size from about 600 to 200 nm. There is little difference in bioavailability for the different lipid to drug ratios. At the same lipid to drug ratio (15% TU loading) the stearic acid containing NLC show a lower bioavailability. This is attributed to the slower degradation and solubilization of these particles compared to the glyceride particles. Different degradation velocities for lipid nanoparticles as a function of the matrix lipid were shown by Olbrich et al.[24–26]

It is known from the literature that in the fed status the bioavailability of Andriol Testocaps® is increased[6,7]. This was confirmed by the obtained in vivo data leading to a bioavailability of about 13,000 units. This bioavailability could be matched by NLC2 and NLC3. Hence, it is possible to replace the Andriol Testocaps® formulation by NLC formulations providing a

Table 6. Short-term stability of the formulations.

Formulation	Day 0		Day 3		1 week		4 weeks		3 months	
PCS size in nm and PI (in brackets)										
NLC1	592 ± 7 (0.15 ± 0.01)		576 ± 12 (0.55 ± 0.05)		Unstable		Unstable		Unstable	
NLC2	176 ± 1 (0.23 ± 0.01)		173 ± 4 (0.23 ± 0.03)		169 ± 1 (0.30 ± 0.17)		175 ± 3 (0.22 ± 0.01)		174 ± 4 (0.23 ± 0.02)	
NLC3	215 ± 3 (0.15 ± 0.01)		228 ± 7 (0.13 ± 0.11)		227 ± 5 (0.13 ± 0.08)		208 ± 1 (0.15 ± 0.02)		232 ± 3 (0.11 ± 0.01)	
NLC4	200 ± 3 (0.21 ± 0.01)		281 ± 8 (0.35 ± 0.23)		Unstable		Unstable		Unstable	
Laser diffractometry size data (LD50 and LD90) in µm										
	LD50	LD90	LD50	LD90	LD50	LD90	LD50	LD90	LD50	LD90
NLC1	0.37	0.91	0.45	1.24	Unstable					
NLC2	0.25	0.40	0.26	0.43	0.28	0.41	0.26	0.44	0.29	0.47
NLC3	0.28	0.48	0.29	0.53	0.30	0.55	0.29	0.51	0.33	0.55
NLC4	0.29	0.54	0.37	0.77	Unstable					

Table 7. Area under the curve after 8 hours of serum testosterone after oral administration of the formulations in male Wistar rats (after Muchow[23]).

Formulation	Bioavailability: AUC after 8 hours (pg·h/mL)
NLC1	10,208
NLC2	12,933
NLC3	13,950
NLC4	11,976
Andriol Testocaps in Tween 80	8,542
Andriol in Testocaps in castor oil/Lauroglycol FCC	13,105

similar bioavailability to Andriol Testocaps® even in the nonfed state.

Conclusions

NLC could be produced with a drug loading of up to 30%, which is superior to the solubility of TU in Andriol Testocaps® (about 10%). TU is obviously present in the particle matrix as molecular dispersion. Sizes can be tailor-made depending on surfactant concentration and production conditions. The glyceride-based NLC with smallest particle size (about 200 nm) could match the bioavailability of Andriol Testocaps® in the fed state. The combination of higher solubility and similar bioavailability opens the perspective to produce an oral formulation, which can be administered in one oral unit (instead of two capsules). Furthermore, it is expected that the difference between nonfed and fed status is reduced, similar to fenofibrate nanoparticles in Tricor®[27].

Declaration of interest

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