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Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles

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Abstract

Solid lipid nanoparticles (SLN) have been prepared from three oil-in-water microemulsions, whose internal phase was constituted of different lipid matrices. The dispersion media were two aqueous solution of trehalose and Pluronic F68 at 2% besides distilled water. SLN were sterilized by autoclaving, were stable during sterilization and maintained a spherical shape and narrow size distribution as confirmed by TEM analysis. SLN dispersions in water did not present nanoparticles larger than 1 μm after storage at 4°C for 1 year; they were freeze-dried after sterilization to obtain dry products. Diazepam was used as model drug to incorporate into SLN, where it was shown by calorimetric analysis to be in amorphous form. © 1997 Elsevier Science B.V.

Keywords: Solid lipid nanoparticles; Sterilization by autoclaving; Freeze-drying; Diazepam; Amorphous form

1. Introduction

Solid lipid nanoparticles (SLN) have been proposed as colloidal drug carriers for most administration routes. SLN as alternative particulate carriers have attracted increasing attention because of the several advantages they offer: their components are bioacceptable and biodegradable; their size is small; their toxicity is lower than that of polymeric nanoparticulates; can be incorporated sufficient amounts of drugs; sustained delivery of drugs is possible. For parenteral administration, the size of SLN is a limiting factor.

For intravenous use of SLN dispersions the presence of large particles have to be controlled. The USP XXIII limits in small volume injections (100 ml or less) the particulate presence to 6000 particles per container equal or greater than 10 μm in diameter.

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Average diameters above 5 μm should in any case be avoided in preparation of SLN for i.v. administration. Thus size control and the avoidance of nanoparticle growth are important considerations in preparing SLN dispersions. Aggregation or modification of nanoparticles may occur during storage, if SLN dispersions are stored in water after sterilization.

Nanoparticle preparation have been developed by a number of research groups; these nanoparticle systems have been preprared from solid lipids, such as glycerides, fatty acids, cholesteryl acetate, using different processes. Müller et al. (1995) produced SLN by high pressure homogenization of a melted lipid dispersed in an aqueous surfactant solution; on cooling to room temperature, the oil droplets solidified and formed SLN. For drug loaded SLN, the drug was dissolved in the melted lipid prior to emulsification. The SLN were physically stable during sterilization by autoclaving. Westesen et al. (1993) proposed two methods to produce SLN: by sonication or by high pressure homogenization; Domb (1995) obtained solid lipospheres by a melt technique; Sjöström and Bergenstål (1992) proposed precipitation from solvent emulsions.

As previously reported (Cavalli et al., 1995a), we prepared SLN by dispersing warm oil-in-water (o/w) microemulsions in a cold aqueous medium under mechanical stirring; this method to obtain SLN requires two steps:

- formulation of warm o/w microemulsion
- formation of the solid nanoparticles by dispersing the warm microemulsion in a cold aqueous medium.

Microemulsions are clear, thermodynamically stable, optically isotropic systems, obtained spontaneously by mixing surfactant, cosurfactant, oil and water (Bourrel and Schecter, 1988). Thus their preparation does not require energy. The simplest representation of the structure of rnicroemulsions is the droplet model with small droplet diameter, generally below 140 nm. In the aqueous medium, SLN form by crystallization of the oil droplets present in the o/w microemulsion; consequently, the nanoparticle size is affected by the composition of the microemulsion system, particularly by the surfactant and cosurfactant used, as well as by the experimental parameters (Cavalli et al., 1996).

SLN dispersions can be freeze-dried to obtain dry products which can easily be stored and reconstituted before use by addition of an aqueous medium. The freeze-drying process may also modify the size and the shape of the SLN.

The first aim of this study was to verify the influence of sterilization by autoclaving as well as of freeze-drying on sizes and stability of drug-free and drug-loaded SLN. The second aim was to evaluate the stablity of sterilized and freeze-dried SLN dispersions during storage for more than one year. Different lipids were chosen to prepare SLN, for comparison of their behaviour after the two processes and during storage over time. Three microemulsions were prepared, using a diglyceride (monostearate monocitrate diglyceride) and two fatty acids (stearic acid and behenic acid) respectively, as oil, while the other components were maintained fixed, to verify the role played by the lipid matrix.

In a preliminary study, using the same three microemulsion formulations, different surfactants were tested to establish their influence on size of nanoparticles; lecithin appeared to be the best performing surfactant, producing small nanoparticles.

Besides water, two aqueous solutions (trehalose 2% and Pluronic F68 2%) were used to disperse SLN, to compare the influence of such solutions on average diameter and polydispersity index of SLN after sterilization and freeze-drying. Trehalose was chosen because it seems to act as cryoprotector, while Pluronic F68 was chosen because it probably plays a role of steric stabilizator of nanoparticles (Schwarz et al., 1994).

The behaviour during sterilization and freeze-drying of drug-loaded SLN was also studied; as model lipophilic drug, diazepam was incorporated into SLN.

2. Materials and methods

2.1. Materials

Stearic acid and behenic acid were from Fluka (Buchs, CH); Acidan N12 (monostearate monoci-

Table 1
Average diameter, polydispersity index and zeta potential of SLN constituted of Acidan N12 (monostearate monocitrate diglyceride), before and after sterilization

		Water	Trehalose 2%	Pluronic F68 2%
Average diameter (nm)	before	60.0	85.0	78.0
	after	65.0	85.0	58.0
Polydispersity index	before	0.23	0.27	0.32
	after	0.22	0.27	0.30
Zeta potential (mV)	before	−46.0	−44.0	−20.5
	after	−44.0	−44.0	−19.5

trate diglyceride) was from Grindsted (Grindsted, Brabrand, Denmark); Epikuron 200 (soya phosphatidylcholine 95%) was a kind gift from Lucas Mayer (Hamburg, Germany); diazepam, and trehalose were from Sigma (St. Louis, MO USA); Pluronic F68 (poloxamer 188, triblock copolymer of polyoxyethylene and polyoxypropylene) was from Serva (Heidelberg, Germany). Taurodeoxycholate sodium salt (TDC) was a kind gift from PCA (Basaluzzo, Italy).

2.2. Preparation of microemulsions

Three o/w microemulsions were prepared. The oil phase (7.53% w/w) was stearic acid (microemulsion S), behenic acid (microemulsion B) or Acidan N12 (microemulsion A); surfactant (3.9% w/w) was Epikuron 200, cosurfactant (13.8% w/w) was taurodeoxycholate (TDC) and the continuous phase (75.32% w/w) was distilled water in all cases.

The chosen lipid was melted, then surfactant, cosurfactant and warm water were added successively. A clear microemulsion was obtained under gentle stirring at a temperature close to the melting point of the lipid used.

For drug-loaded SLN, diazepam was added to the melted lipid before the other components The amount of drug incorporated was 10% of the internal phase.

2.3. Preparation of SLN

SLN (A,B,S) were obtained by dispersing the warm o/w microemulsions in a cold aqueous medium (about 2°C) under mechanical stirring at a ratio of 1:25 (microemulsion/aqueous medium). The aqueous media used were aqueous solutions of trehalose or Pluronic F68 at 2%, or distilled water.

The SLN dispersion was then washed twice with the dispersion medium used by diaultrafiltration with a TCF2 system (Amicon-Grace, Danvers, USA) using a Diaflo YM100 membrane (cut off 100 000 Da) in order to eliminate a large proportion of the cosurfactant molecules used to obtain the microemulsion.

2.4. Sterilization and freeze-drying of SLN

SLN dispersed in the different dispersion media were autoclaved at 121°C, 2 bar, for 15 min following the European Pharmacopoeia II. SLN were freeze-dried before or after sterilization using a Modulyo freeze-dryer (Edwards Crawley, UK), continuing the process for 36 h.

2.5. Characterization of SLN

2.5.1. Photon correlation spectroscopy

Particle size was determined by photon correlation spectroscopy (PCS) using an N4 Coulter at fixed angle of 90° and at a temperature of 25°C.

The average diameter and polydispersion index of SLN were determined before and after sterilization and before and after freeze-drying. Each value was the average of ten measurements.

The polydispersion index is a measure of the distribution of nanoparticies (Koppel, 1972).

The stability of sterilized SLN was monitored over time, measuring the size of nanoparticles

Table 2
Average diameter, polydispersity index and zeta potential of SLN constituted of behenic acid, before and after sterilization

		Water	Trehalose 2%	Pluronic F68 2%
Average diameter (nm)	before	70.0	90.0	85.0
	after	135.0	150.0	60.0
Polydispersity index	before	0.21	0.26	0.36
	after	0.22	0.27	0.30
Zeta potential (mV)	before	−48.0	−44.0	−20.5
	after	−36.0	−44.0	−19.5

after fixed storage times (1 day, 1, 2, 4, 6, 8, 10, 12 months); the SLN dispersions were stored at 4°C.

2.5.2. Trasmission electron microscopy

Trasmission electron microscopy (TEM) analysis was performed using a Philips CM10 instrument. The SLN dispersions were stained with a 2% solution of osmium tetraoxide.

2.5.3. Zeta potential analysis

The electrophoretic mobility and zeta potential measurements were determined using a Delsa 440 (Coulter) instrument; SLN dispersions were diluted 1:5 with bidistilled water before analysis.

2.5.4. Differential scanning calorimetry

A differential scanning calorimeter DSC 7 (Perkin Elmer) equipped with an instrument controller Tac 7/DX (Perkin Elmer) was used. The instrument was calibrated with indium for melting point and heat of fusion. A heating rate of 20°C/min was employed throughout the analysis in the 25–200°C temperature range. Standard aluminum sample pans (Perkin Elmer) were used for all the samples; an empty pan was used as reference. The thermal behaviour was studied under a nitrogen purge; triplicate run were carried out on each sample to check reproducibility.

2.6. Preparation of mixtures for the thermal analysis

Two mixtures of the components of the SLN, in the same molar ratio as in microemulsion, were investigated by calorimetric analysis. The mixtures prepared were:

- stearic acid (0.7 mM) and diazepam (0.035 mM)
- stearic acid (0.7 mM), Epikuron 200 (0.14 mM) and diazepain (0.035 mM)

The mixtures were subjected to the same thermal cycle as the SLN; after melting, the mixtures were dispersed in water, washed, filtered and dessicated.

2.7. Determination of diazepam incorporated into SLN

The diazepam-loaded SLN were dissolved in methanol and analysed directly.

The amounts of diazepam incorporated into the SLN, whether sterilized or not, were determined by HPLC (Perkin Elmer Binary LC Pump 250 liquid chromatograph, Bio-Rad column ODS 5 cm × 4.6 mm). The eluent was methanol/water 60:40 v/v. The analysis was run at a flow rate of 0.6 ml/min with the UV detector operating at 235 nm. (Waters Assoc., 1973).

3. Results and discussion

3.1. Sterilization of SLN with different lipid matrices

For intravenous and ocular administration SLN must be sterile. The high temperature reached during sterilization by autoclaving presumably causes a hot o/w microemulsion to form in the autoclave, and probably modifies the size of the hot nanodroplets. On subsequent slow cooling, the SLN reformed, but some nanodroplets

Table 3
Average diameter, polydispersity index and zeta potential of SLN constituted of stearic acid, before and after sterilization

		Water	Trehalose 2%	Pluronic F68 2%
Average diameter (nm)	before	55.0	56.5	76.0
	after	110.0	129.0	169.0
Polydispersity index	before	0.20	0.27	0.30
	after	0.25	0.29	0.30
Zeta potential (mV)	before	−46.5	−44. 0	−20.5
	after	−37.0	−44.0	−23.0

may coalesce, producing larger SLN than the initial ones. Since SLN are washed before sterilization, amounts of surfactant and cosurfactant present in the hot system are smaller, so that the nanodroplets may be not sufficiently stabilized.

Before sterilization, the average diameter of nanoparticles A, B and S dispersed in water ranged from 55 nm to 70 nm and the polydispersity index from 0.20 to 0.23, according to the lipid matrix used.

The average diameter and polydispersity values after sterilization are reported in Tables 1–3. After sterilization, the average diameter of the A nanoparticles dispersed in water or trehalose increased, but to a negligible extent, while in Pluronic F38, there was a decrease (Table 1). The polydispersity values varied slightly after sterilization, indicating that the change in the population distribution was only small.

After sterilization, the S nonoparticles, constituted of stearic acid, showed an increase in all dispersion media, still being in the colloidal range. The increase of polydispersity was slight.

In the case of the B nanoparticles, the increase was obvious only when dispersed in water, whereas there was no increase in trehalose or in Pluronic F38 as shown in Table 2.

Probably, there being lower moles of behenic acid (0.64 mM) than of stearic acid (0.70 mM), the dissolved Pluronic molecules might protect the nanoparticles, avoiding coalescence.

After sterilization, all dispersions of SLN presented nanoparticles in the colloidal range.

The sterilized SLN were also analysed by Trasmission Electron Microscopy. All TEM mi-

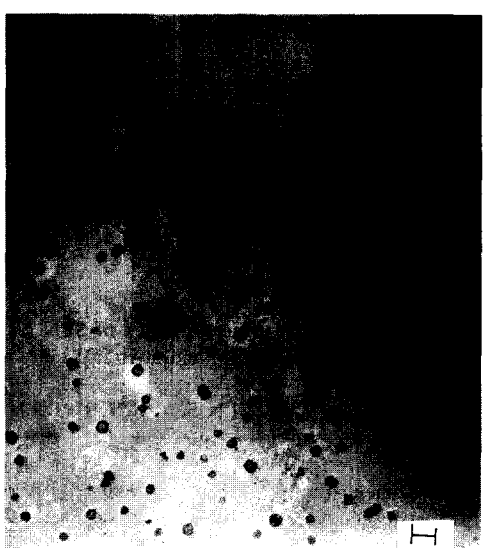

Fig. 1. Transmission Electron Microscopy photomicrograph of SLN S dispersed in water after one month sterilization. Bar = 200 nm.

Table 4
Average diameter and polydispersity index of SLN containing diazepam, before and after sterilization

Lipid used	Average diameter (nm)	Polydispersity index
Stearic acid		
Before	78.5	0.16
After	120.0	0.20
Behenic acid		
Before	86.0	0.15
After	116.0	0.18
Acidan N12		
Before	70.0	0.18
After	75.5	0.16

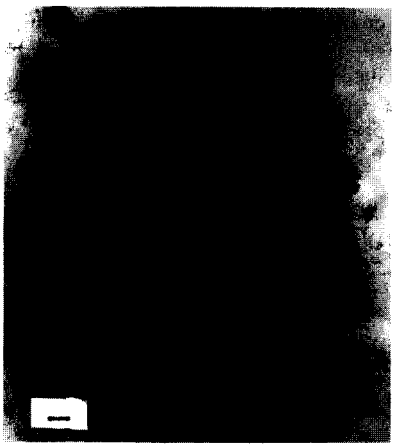

Fig. 2. Transmission Electron Microscopy photomicrograph of SLN S containing diazepam dispersed in water after sterilization. Bar = 100 nm.

crographs confirmed that the colloidal size and spherical shape of SLN remained after sterilization, and showed the narrow size distribution of the nanoparticle dispersion.

Fig. 1 shows the micrograph of the S nanoparticles dispersed in water one month after sterilization.

After sterilization, the drug-loaded SLN dispersed in water behaved as the drug-free SLN.

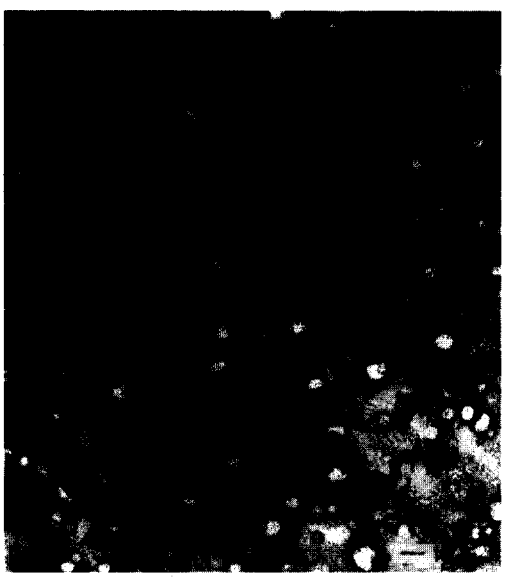

Fig. 3. Sterilized SLN constitued of behenic acid after 15 months at 4°C. Bar = 100 nm.

Table 5
Average diameter and polydispersity index of SLN dispersed in water, before and after freeze-drying

	SLN S	SLN B	SLN A
Before freeze-drying			
Average diameter (nm)	55.0	70.0	60.0
Polydispersity index	0.20	0.21	0.23
After freeze-drying			
Average diameter (mn)	195.0	225.0	280.0
Polydispersity index	0.28	0.40	0.17

The average diameter and polydispersity index of SLN containing diazepam are in Table 4; a slight increase in size was observed for all the lipid matrices.

The polydispersity values indicate a narrow size distribution. The drug-loaded SLN maintained their spherical shape after sterilization, as shown by TEM micrograph (Fig. 2).

The zeta potential of SLN dispersed in water decreased after sterilization, particularly for the nanoparticles constituted of fatty acids. In the case of SLN having Acidan N12 as lipid matrix the difference of zeta potential values before and after sterilization was smaller, as shown in Table 1. In the trehalose solution the zeta potential of SLN did not change before and after sterilization, while in Pluronic F68, there was only a small decrease.

The stability over time after sterilization was monitored only for the SLN dispersed in water, to verify the effect of storage conditions on SLN size.

Table 6
Average diameter and polydispersity index of SLN dispersed in trehalose solution (2%), before and after freeze-drying

	SLN S	SLN B	SLN A
Before freeze-drying			
Average diameter (nm)	56.5	90.0	185.0
Polydispersity index	0.27	0.26	0.27
After freeze-drying			
Average diameter (nm)	240.0	285.0	390.0
Polydispersity index	0.31	0.40	0.22

Table 7
Average diameter and polydispersity index of SLN dispersed in Pluronic F68 solution (2%), before and after freeze-drying

	SLN S	SLN B	SLN A
Before freeze-drying			
Average diameter (nm)	76.0	85.0	78.0
Polydispersity index	0.30	0.36	0.32
After freeze-drying			
Averaged diameter (nm)	325.0	235.0	385.0
Polydispersity index	0.35	0.39	0.40

All dispersions appeared uniform, with no separation between aqueous phase and nanoparticles after more than 1 year (see Fig. 3). The average diameter and polydispersity index values increased over time; after 1 year, the average diameters were 350 nm, 120 nm and 450 nm, the polydispersity indices were 0.35, 0.38 and 0.33, respectively, for SLN A, B and S. No particles larger than 1 μm could be observed. For the first 6 months the increase was neglegible, so it could be possible improve the stability over time by adding some dispersing agent or improving the microemulsion composition.

3.2. Freeze-drying of SLN after sterilization

In this study we sterilized the dispersions of SLN by autoclaving successively freeze-drying them, in order to examine the possible difference in SLN size. Sterile SLN can also be obtained by a sterilizing filtration of the hot o/w microemulsion, which can be freeze-dried after dispersion in aseptic conditions.

All freeze-dried SLN were readily redispersed in water under mechanical stirring at the dispersion ratio 1:25, also after a storage of one year. Size measurements of reconstituted SLN by PCS showed an increase in their average diameter and polydispersion index (Tables 5–7).

All lipid matrices used formed larger SLN with a wider size distribution after freeze-drying, probably due to the presence of aggregates between nanoparticles.

The conditions of the freeze-drying process and the removal of the water probably promoted aggregation among SLN; the amount of cryoprotector used during the dispersion was only 2%, probably too low to protect the nanoparticles during this process.

To confirm this hypothesis, we dispersed S nanoparticles in a 15% trehalose solution; this dispersion was freeze-dried and then redispersed, obtaining SLN with an average diameter of about 100 nm and a polydispersity index of 0.25. This experiment indicate that a high percentage of cryoprotector can improve results.

Table 8
Percentages of diazepam incorporated into SLN

Lipid used	% Diazepam into SLN
Stearic acid	1.88
Behenic acid	2.25
Acidan N12	1.20

3.3. Percentages of diazepam in SLN

The percentages of diazepam incorporated into SLN are reported in Table 8.

The amounts incorporated do not change after sterilization, indicating that the presence of the drug does not influence the stability of SLN during the process. The incorporation varies according to the lipid matrices. The diglyceride is less suitable for the drug incorporation.

3.4. Calorimetric analysis of drug-loaded SLN

The thermal analysis was carried out on drug-free and drug-loaded SLN and on melted mixtures containing stearic acid.

The melting peak of diazepam disappears in the thermogram of drug-loaded SLN (see Fig. 4) while it is always present in the mixtures. This suggests that diazepam is present in its amorphous form in SLN.

As previously noted for nifedipine and phenothiazine (Cavalli et al., 1995b), when the microemulsions are quenched, the drug molecules dispersed in the lipid phase cannot nucleate as crystals. This behaviour should improve the solubility of diazepam in water, and consequently may offer a possible interesting development in therapy.

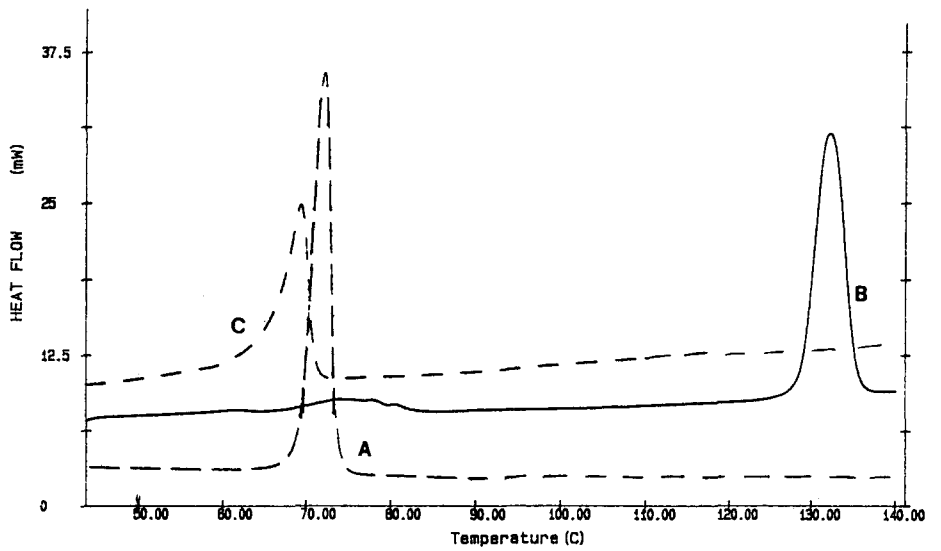

Fig. 4. DSC thermograms of: (A) stearic acid, (B) crystalline diazepam, (C) SLN S containing diazepam.

4. Conclusions

SLN can be sterilized by autoclaving, maintaining an almost spherical shape, without any significant increase in size or nanoparticle distribution; this is an advantage in comparison with some polymeric carriers.

The stability of sterilized SLN over time apparently depends on the lipid matrix, but the dispersing media may also play an important role.

The presence of diazepam in SLN in its amorphous form is interesting; by changing the composition of the microemulsions it should be possible to improve the amount of drug incorporated into SLN; in any case, the presence of the drug did not influence the stability of SLN.

Acknowledgements

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