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Preparation and In Vivo Toxicity Study of Solid Lipid Microparticles as Carrier for Pulmonary Administration

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ABSTRACT

The purpose of this research was to investigate the effects of processing conditions on the characteristics of solid lipid microparticles (SLM) with a potential application as carriers for pulmonary administration. Compritol (5.0% wt/wt) SLM dispersions were prepared by rotor-stator homogenization, at different surfactant concentrations and emulsification times. The SLM were characterized, in terms of morphology and size, after lyophilization and sterilization by autoclaving process. In vivo assessment was carried out in rats by intratracheal instillation of either placebo or SLM dispersion, and by bronchoalveolar lavage for cytological analysis. Mean particle size of 4 to 5 μm was achieved using 0.3% and 0.4% (wt/wt) of emulsifier (Poloxamer 188) and emulsification times of 2 and 5 minutes. The particles showed spherical shape and smooth surface. The morphology of microparticles, the size, and the size distribution were not substantially modified after lyophilization and sterilization. Total cell counts showed no significant differences between placebo and SLM 0.5% or 2.5% groups. Regarding cytology, percentage of polymorphonuclear neutrophils and macrophages did not significantly differ between groups. These results suggest that a single intratracheal administration of the SLMs does not induce a significant inflammatory airway response in rats and that the SLMs might be a potential carrier for encapsulated drug via the pulmonary route.

KEYWORDS: solid lipid microparticles, pulmonary administration, lyophilization, sterilization, pulmonary toxicity

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INTRODUCTION

In recent years, biocompatible lipid micro- and nanoparticles have been reported as potential drug carrier systems as alternative materials to polymers.[1-3] Solid lipid particles combine several advantages and avoid the disadvantages of other colloidal carriers. The following are positive features of the potential use of solid lipid particles as drug carrier systems:

- They offer the possibility of controlled drug release and drug targeting.[4]
- They provide protection of incorporated active compounds against degradation.
- Their solid matrix is composed of physiological and well-tolerated lipids.
- They allow for hydrophilic and/or hydrophobic drugs to be incorporated.[5,6]

The drug solubility and miscibility in melted lipid, chemical and physical structure of lipid materials, and their polymorphic state determine the loading capacity of drug in the lipid particles.[7] The amount of drug encapsulated can vary from 1% to 5% for hydrophilic compounds[8,9] and up to 80% for lipophilic compounds.[5,10]

Solid microparticles in dispersions are usually obtained using a melt dispersion method or a solvent evaporation method[11]; the advantage in the melt method is that no organic solvents are needed.

Solid lipid particles have been proposed as a colloidal drug carrier therapeutic system for different administration routes such as oral, topical,[12,13] ophthalmic, subcutaneous and intramuscular injection,[9] and particularly for parenteral administration.[14,15]

Several sustained-release systems that include liposome[16-18] and other biodegradable microspheres[19,20] have been investigated as potential pulmonary carriers.

Until now the solid lipid microparticle (SLM) system has not yet been fully exploited for pulmonary drug delivery; little has been published in this area. The solid lipid particles might be used for pulmonary delivery in aqueous dispersions by nebulization or in dry powder inhalers.[7]

The lungs can provide a very effective means of delivery for many drugs. Medical conditions such as asthma, chronic obstructive pulmonary disease (COPD), or cystic fibrosis have traditionally been treated by inhaled drug delivery to the airways.

After administration to the airways, lipids may be eliminated by the common mechanisms for removal of carriers from the lungs, which are mucociliary transport, phagocytosis, or systemic absorption.[21]

The particle size and the potential toxicity of excipients are 2 of the critical factors that limit SLM use for pulmonary administration.[7,22]

The broadest and deepest penetration of particles into the airways and their deposition in the peripheral regions are achieved when the particle size ranges between 1 and 5 μm.[23]

The excipients must be physiologically acceptable, biodegradable, and nonimmugenic and might not induce inflammatory and alloreactive responses.[24,25]

Solid lipid particles are well tolerated in living systems because they are made from physiological or physiologically related materials, therefore metabolic pathways exist; this finding is supported by in vitro studies of cytotoxicity and biodegradation.[26,27] However, only a few in vivo studies have been performed as yet; and, in particular, toxicity studies after pulmonary administration should be investigated.

Among the screening methods allowing assessment of the presence of an inflammatory process occurring within the lower airways figures the cytological analysis of bronchoalveolar lavage fluid (BALF). By careful instillation of fluid into the airways, epithelial lining fluid and cellular components might be successfully recovered and analyzed. An increase of the total cell number in lavage fluid and modifications of the cellular population (eg, increase in polymorphonuclear neutrophils, in macrophages, etc) are considered as unspecific, but sensitive markers of an inflammatory reaction occurring within the lavaged lung parts.

Sterilization of microparticles should be taken into account in the case of pulmonary or parenteral administration. As reported in literature, the lipid particles are physically stable during sterilization by autoclaving.[28,29,4]

The aims of this study were the following:

- investigation of the optimal conditions to produce SLM with a suitable diameter for penetration into the lower airways and to study the parameters affecting their preparation process.
- characterization in terms of morphology (shape and surface) and size of SLM produced.
- determination of SLM stability after lyophilization and sterilization by autoclaving processes.
- assessment of the acute pulmonary toxicity of SLM in vivo by analyzing BALF after intratracheal instillation of SLM dispersions in rats.

MATERIALS AND METHODS

Materials

Compritol 888 ATO (Glycerol behenate) was provided by Gattefossé (St Priest, Cedex, France); this material has a melt point of 72°C and is a mixture of 12% to 18% mono-, 52% to 54% di-, and 28% to 32% triglycerides of behenic acid (more than 87% of the fatty acid fraction). Lutrol F68 (Poloxamer 188) was obtained from BASF AG (Ludwigshafen, Germany). PBS (Phosphate Buffer Saline) was purchased from Merck (Darmstadt, Germany).

Solid Lipid Microparticle Preparation

SLM were obtained by oil in water (o/w) emulsification employing the phase inversion technique.[30,31]

Compritol was used as lipophilic component and Poloxamer as emulsifying agent.

The emulsions consisting of 5.0% (wt/wt) Compritol, different concentrations of Poloxamer (0.3%, 0.4%, 0.5%, 0.6%, 0.9% wt/wt) and water up to 100 g were prepared.

Compritol and the surfactant were heated to 90°C and hot purified water at the same temperature was slowly added to oily phase. The emulsions were prepared by Silverson L4R mixer (EJ Payne Ltd, London, UK), emulsifying at 6200 rpm for different times (2, 5, 10, and 15 minutes). The emulsions obtained were cooled at room temperature under magnetic stirring until solidification of the microparticles occurred.

Table 1 shows the formulations prepared. Each formulation was prepared in triplicate.

Solid Lipid Microparticle Lyophilization

Formulation obtained by using 0.4% (wt/wt) of Poloxamer and 5 minutes of emulsification time (SLM 2b) was chosen, on the basis of particle size results, for next steps of the study.

Table 1. Effects of Different Poloxamer Concentrations and Emulsification Times on SLM Mean Diameter*

Formulation	Poloxamer Percentage (wt/wt)	Emulsification Time (minutes)	Particles Mean Diameter (μm) ± SD
SLM 1a	0.3	2	4.9 ± 1.5
SLM 1b	0.3	5	4.5 ± 1.0
SLM 2a	0.4	2	4.1 ± 1.1
SLM 2b	0.4	5	4.4 ± 0.9
SLM 3a	0.5	2	8.2 ± 1.5
SLM 3b	0.5	5	8.1 ± 0.3
SLM 4a	0.6	2	8.9 ± 0.4
SLM 4b	0.6	5	10.2 ± 0.8
SLM 5a	0.9	2	10.3 ± 2.5
SLM 5b	0.9	5	12.9 ± 0.9

*SLM indicates solid lipid microparticles. Results expressed are the mean of 4 measures ± SD.

Aliquots of SLM 2b diluted with water were rapidly frozen in an ethanolic bath at -25°C for 15 minutes. Lyophilization was carried out at -25°C under vacuum (1.10-3 mbar) for 30 hours in Heto-Holten apparatus (Gydevang, Denmark).[32]

Solid Lipid Microparticle Sterilization

The SLM 2b lyophilized was dispersed at different concentrations (0.5%, 1.0%, 1.5%, 2.0%, and 2.5% wt/wt) in PBS isotonic buffer (pH 7.4) and Poloxamer (2.0% wt/wt); after sonication for 5 minutes the dispersions were autoclaved at 121°C, 1 bar, for 20 minutes.

Poloxamer 2.0% has been used to obtain the optimal redispersion of SLM 2b.

Solid Lipid Microparticle Characterization

Particle Size Analysis

Particle size analysis was carried out on SLM after production and on SLM 2b after lyophilization and sterilization.

Particle size analyses were performed using suspensions of SLM.

The particle size and particle size distribution were determined by laser diffractometer Mastersizer 2000 with the Hydrosizer 2000S module (LD) (Malvern Instruments, Worcestershire, UK).

The sample was added to the water under magnetic stirring (2500 rpm) until an obscuration rate of 5% to 18% was reached. Optical properties of the sample were defined as follows: refractive index 1.460 and absorption 0.00 (similar to the particles named Intralipid in the Malvern software).

The LD data were evaluated using volume distribution to detect submicronic particles as well as possible particle aggregates.

Three suspensions were prepared for each sample tested; each suspension was analyzed 4 times.

Scanning Electronic Microscopy

The morphological examination (shape and surface characteristics) of SLM was performed by scanning electron microscopy (SEM), model DSM 962 (Carl Zeiss Inc, Oberkochen, Germany). Samples of SLM were placed on double-sided tape, which had previously been secured to aluminum stubs. The samples were then analyzed at 20 kV acceleration voltage after gold sputtering, under an argon atmosphere.

In Vivo Studies

On the basis of the results of particle size analysis, the formulation SLM 2b was chosen for the in vivo studies.

The animals used in these experiments were male Sprague-Dawley rats (n = 54), weighing 250 to 350 g.

The Animal Ethics Committee of the University of Liège (Liège, Belgium) approved the experimental protocol.

Intratracheal Instillation of Placebo or Solid Lipid Microparticle Suspensions

All animals were weighed prior to anesthesia. The rats were anesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). Intratracheal instillation was performed using a 0.5-mm inner diameter flexible catheter, which was introduced via the oral cavity and the larynx into the distal part of the trachea. Control rats (n = 18) were instilled with 100 μL of PBS solution, whereas treated rats were instilled with 100 μL of SLM dispersion at 0.5% (wt/wt) (n=18) and 2.5% (wt/wt) (n = 18). In order to favor lower airway penetration of the instilled solution, all

animals were placed in dorsal recumbency during recovery of anesthesia.

Bronchoalveolar Lavage

After being weighed, rats were euthanized by intraperitoneal injection of an overdose of pentobarbital 24 (T24), 48 (T48), or 72 (T72) hours postinstillation.

Lungs were carefully removed from the thoracic cavity in order to perform bronchoalveolar lavage (BAL). A rigid catheter was inserted into the trachea, and a tight ligature was placed around the trachea and the catheter. Fifteen milliliters of sterile saline (0.9%) were then slowly instilled through the catheter into lung lobes and were aspirated by gentle suction.

Collected BALF was kept at 4°C until analysis, which was performed within 6 hours of collection. Total cell count was performed using a Thoma cell on which 20 μL of colorated BALF (Türck solution, 1:1) was placed. Nucleated cells were counted under light microscopy, and 4 counts were performed per rat. Cytological analysis was performed after cytospin centrifugation of BALF and Giemsa staining. At least a hundred nucleated cells were counted, and a percentage of alveolar macrophages [M], lymphocytes [L], and polymorphonuclear neutrophils [PMN] was established. Knowing the total cell number, the number of cell types per milliliter of BALF could be calculated.

Data were analyzed by a 2-way analysis of variance (ANOVA) in order to value the effect of particle administration (ie, placebo, SLM 0.5%, or SLM 2.5%) and time after instillation (ie, 24, 48, or 72 hours postinstillation); $P < .05$ was considered to be significant.

RESULTS AND DISCUSSION

The effects of production conditions on the SLM characteristics were investigated.

Table 1 shows the formulations obtained at different surfactant concentrations and emulsification times; moreover, particle size results are reported.

The choice of the emulsifier and its concentrations greatly affect the particle size of solid particles.[33]

To evaluate the influence of the surfactant, 5 different concentrations of Poloxamer 188 (0.3%, 0.4%, 0.5%, 0.6%, and 0.9% wt/wt) were used.

The results demonstrate that with 0.3% and 0.4% (wt/wt) of Poloxamer, the mean particle size remains substantially unchanged. Increasing the Poloxamer concentration determines a significant change of the mean particle diameter (~8.0 μm for 0.5% wt/wt Poloxamer concentration and ~10.0 μm for both 0.6% and 0.9% wt/wt).

A possible explanation for this particular behavior is the relatively small amount of emulsifier used compared with the concentrations generally used (2.0%-5.0% wt/wt). The emulsifier concentrations utilized may have been inefficient to reduce the surface tension and facilitate the particle partition during the homogenization process.

To evaluate the influence of the emulsification time, the SLM dispersions were prepared at 4 different times (ie, 2, 5, 10, and 15 minutes).

For all the formulations, the emulsification time is negligible when 2 or 5 minutes are used. However, significant differences in particle size are observed after 10 and 15 minutes; in fact, from SLM 1a to SLM 5b, mean diameter values range from 9 to 15 μm and 16 to 19 μm, respectively. Prolonged time of emulsification leads to an increase in particle size due to particle coalescence resulting from the high kinetic energy of the particles.[34]

On the basis of these particle size results, the formulations 1a, 2a, 1b, and 2b show the dimensions close to the size required. Among these formulations, SLM 2b was chosen as the example for the following steps.

The effects of lyophilization and sterilization processes on the characteristics of SLM 2b in terms of size and morphology were studied.

In order to examine the possible differences in SLM size, the particle size was determined after SLM dispersion manufacturing, before and after lyophilization, and before and after sterilization.

The lyophilization and the sterilization by autoclaving do not change significantly the mean diameter of SLM. As shown in Figure 1 the mean particle size (<5 μm) after lyophilization remains substantially unchanged. However, after sterilization, the particle size distribution changes, with a reduction of aggregates above 10 μm and an increase in the particle population with diameter under 2 μm. Poloxamer seems to protect the SLM by acting as a steric stabilizer during lyophilization and therefore avoiding coalescence of SLM during autoclaving processes.[15]

Figure 2 reports the SLM distribution percentage of population with diameter between 0.1 and 6.0 μm at different concentrations of SLM (0.5%, 1.0%, 1.5%, 2.0%, and 2.5% wt/wt) in PBS after sterilization.

The 0.5% to 2.0% (wt/wt) SLM dispersions are constituted of about 81% of particles with diameter within 0.1 μm and 6.0 μm; 15% of particles have mean size above 6.0 μm; and only 4% of particles show a diameter of about 0.01 to 0.1 μm (data not shown).

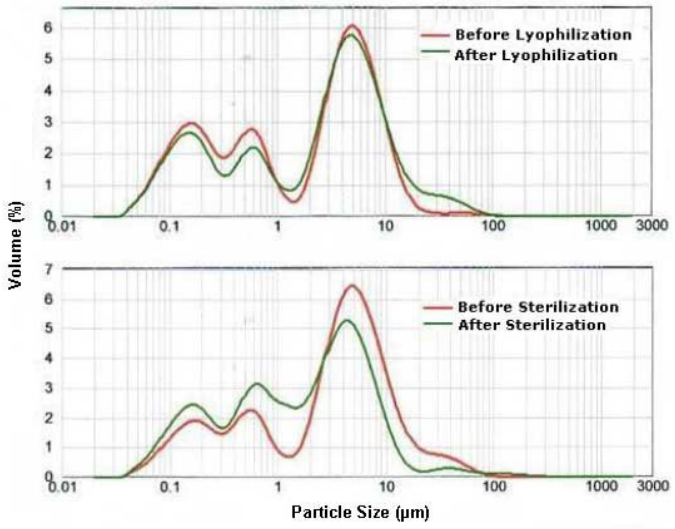

Figure 1. Particle size distributions of SLM 2b before and after lyophilization and sterilization.

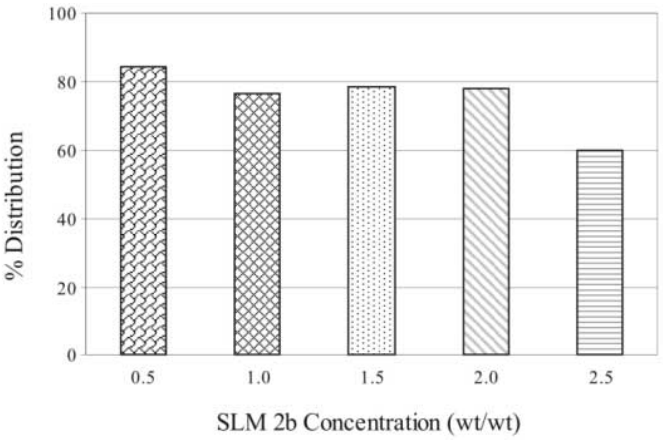

Figure 2. Percentage SLM 2b distribution (in volume) with diameter between 0.1 and 6.0 µm in dependence of SLM concentration after sterilization.

In the 2.5% (wt/wt) SLM dispersions, the particles with diameter of 0.1 to 6.0 µm and less than 0.1 µm correspond to 60.6% and 1.7%, respectively; particles with dimensions above 6.0 µm are 37.7% of the population.

Figures 3A-B show the morphology of SLM 2b chosen as the example.

SEM photomicrograph of SLM 2b after manufacturing shows that the microparticles have a spherical shape and a smooth surface (Figure 3A). The lyophilization and the sterilization processes do not influence significantly the morphological characteristics of SLM (Figure 3B).

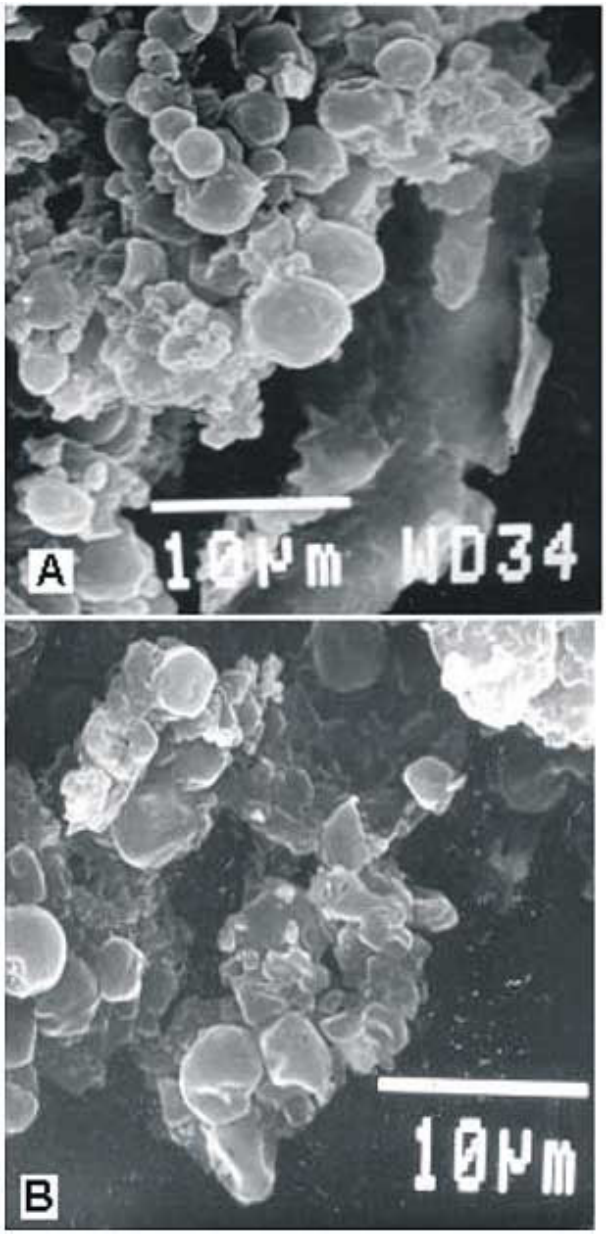

Figure 3. SEM photomicrographs of SLM 2b: (A) after preparation and (B) after sterilization.

The in vivo acute pulmonary toxicity of SLM was investigated.

The inflammatory potential of particles produced was assessed by the total number of inflammatory cells (ie, essentially neutrophils and macrophages) in the BALF of rats that underwent SLM instillations.

There were no macroscopically detectable differences between placebo- and SLM-treated animal lungs. Furthermore, pre-instillation and pre-euthanasia body weight did not differ between groups (data not shown), suggesting that food and water intake was not significantly altered between placebo- and SLM-treated rats.

Table 2. Bronchoalveolar Lavage Total Cell Count and Cytology (Percentage of Macrophages, Lymphocytes, and Polymorphonuclear Neutrophils) of Rats at 24, 48, or 72 Hours After an Intratracheal Instillation of Either 100 µL PBS (Controls), 100 µL of SLM 2b at 0.5% (wt/vol), or 100 µL of SLM 2b at 2.5% (wt/vol)*

Time After Instillation	Variable Analyzed	Controls	SLM 2b 0.5% (wt/vol)	SLM 2b 2.5% (wt/vol)
24 hours		n = 6	n = 6	n = 6
	TC (cells/mL)	62 500 ± 45 000	32 500 ± 20 000	33 800 ± 10 600
	% M	92 ± 11	93 ± 5	98 ± 2
	% L	0	0	0
	% PMN	8 ± 11	7 ± 5	2 ± 2
48 hours		n = 6	n = 6	n = 6
	TC (cells/mL)	67 700 ± 29 500	58 600 ± 20 200	67 700 ± 19 500 †
	% M	90 ± 10	98 ± 3	97 ± 3
	% L	0	0	0
	% PMN	10 ± 10	2 ± 3	4 ± 3
72 hours		n = 6	n = 6	n = 6
	TC (cells/mL)	57 200 ± 24 000	49 500 ± 34 800	150 000 ± 77 400†
	% M	98 ± 2	95 ± 6	92 ± 7
	% L	0	0	1 ± 1
	% PMN	2 ± 2	2 ± 2	7 ± 7

*PBS indicates phosphate buffer solution; SLM, solid lipid microparticles; TC, total cell count; M, percentage of macrophages; L, percentage of lymphocytes; and PMN, polymorphonuclear neutrophils. Data are shown as means ± SD.
†Significantly different from respective T24-value; $P < .05$.

Table 2 shows results (mean ± SD) expressed as both total BALF total cell count (TC, cells/mL) and cytology (percentage M, L, and PMN) of control and SLM-treated rats euthanized 24, 48, and 72 hours postinstillation of 100 µL SLM 2b.

Total BALF cell count is significantly higher at T48 and T72 than at T24 in the SLM 2.5% group, however, without being significantly different from control rats, possibly due in part to an important variability within controls. This increase could indicate a macrophage stimulation and influx further to this important charge of SLM at 2.5%, as well as to a very slight neutrophilic response. However, a slight, but nonsignificant increase of PMN percentage was also noted in the 48-hour control group, suggesting that the procedure of intratracheal instillation might also have contributed to this slight airway irritation.

These screening results suggest that a single SLM instillation at 0.5% and 2.5% does not induce significant airway inflammation in rats and seems to be tolerated by the lower airways. It will however be important to assess the tolerance of those SLM when repeated administrations occur. In this case, a precise estimation of the awaited deposited concentration of SLM will be necessary and further analysis of BALF should be performed, allowing for detection of signs of macrophage or neutrophil activation or migration, rather than limiting the tolerance study to a screening of BALF inflammatory cells. In the case of repeated or chronic SLM administration, the assessment of a functional lung response would also be meaningful.

CONCLUSION

The proposed emulsification method can be used for the preparation of SLM suitable for pulmonary delivery by a 1-step process.

SLM might be a potential carrier for encapsulated drugs to be given by pulmonary route for local (eg, antiasthma, antimicrobial) or systemic therapy.

However, as this drug carrier is especially interesting for long-term treatments, chronic administrations of SLM need to be performed in the future.

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