European Journal of Pharmaceutics and Biopharmaceutics 79 (2011) 189–196

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Development of topotecan loaded lipid nanoparticles for chemical stabilization and prolonged release

L.G. Souza [a], E.J. Silva [a], A.L.L. Martins [a], M.F. Mota [b], R.C. Braga [c], E.M. Lima [a], M.C. Valadares [b], S.F. Taveira [a], R.N. Marreto [a,*]

[a] Laboratory of Pharmaceutical Technology, Federal University of Goiás, Goiânia, Brazil
[b] Laboratory of Cellular Pharmacology and Toxicology, Federal University of Goiás, Goiânia, Brazil
[c] LaBioCon, Federal University of Goiás, Goiânia, Brazil

ARTICLE INFO

Article history:
Received 30 November 2010
Accepted in revised form 15 February 2011
Available online 23 February 2011

Keywords:
Microemulsion
Nanoparticles
Topotecan
Lipid matrix
Chemical degradation
Mass spectrometry

ABSTRACT

Topotecan is an important cytotoxic drug that has gained broad acceptance in clinical use for the treatment of refractory ovarian and small-cell lung cancer. The lactone active form of topotecan can be hydrolyzed *in vivo*, decreasing the drug's therapeutic efficacy. Lipid encapsulation may promote *in vivo* stabilization by removing topotecan from aqueous media. Earlier reports of topotecan lipid nanoencapsulation have focused on liposomal encapsulation; however, the higher stability and cost-effectiveness of solid lipid nanoparticles (SLN) highlight the potential of these nanoparticles as an advantageous carrier for topotecan. The initial motivation for this work was to develop, for the first time, solid lipid nanoparticles and nanostructured lipid carriers (NLC) with a high drug loading for topotecan. A microemulsion technique was employed to prepare SLNs and NLCs and produced homogeneous, small size, negatively charged lipid nanoparticles with high entrapment efficiency and satisfactory drug loading. However, low recovery of topotecan was observed when the microemulsion temperature was high and in order to obtain high quality nanoparticles, and precise control of the microemulsion temperature is critical. Nanoencapsulation sustained topotecan release and improved its chemical stability and cytotoxicity. Surprisingly, there were no significant differences between the NLCs and SLNs, and both are potential carriers for topotecan delivery.

1. Introduction

Topotecan (TPT) is a water-soluble derivate of camptothecin and a topoisomerase I inhibitor [1,2]. TPT is routinely used by clinicians for the treatment of refractory ovarian and small-cell lung cancer [3]. Under neutral or alkaline conditions, the TPT α-hydroxylactone moiety undergoes rapid pH-dependent hydrolysis, leading to the less active carboxylate form [4,5]. Due to this chemical degradation, the antitumor activity of this compound rapidly decreases following dissolution in aqueous media [6].

The lactone moiety of camptothecin drugs can be stabilized by encapsulation in lipid bilayer vesicles, because lactone intercalates between the lipid acyl chains, shielding it from the aqueous media [6]. However, as a hydrophilic camptothecin, TPT displays reduced binding affinity for lipid bilayers [6]. To circumvent this limitation, TPT encapsulation in the aqueous acidic environment of liposomes has been reported by several authors [4,7–12] and was considered to be a promising strategy for the stabilization of TPT. Besides chemical protection, liposomal encapsulation enables increased antitumor efficacy and decreased toxicity due to higher accumulation of TPT in tumors [10]. In spite of these advantages, liposomes display some storage and drug leakage problems and a relatively high cost for large-scale production [13]. Stable formulations are required to take full advantage of the more specific drug distribution into solid tumors (EPR effect) and to maximize cell exposure [12]. The use of solid lipids in nanoparticle preparations is a very attractive alternative for overcoming some of the limitations associated with liposomes and has attracted increasing attention to the possible use of solid lipid nanoparticles (SLN) and related carriers since the 1990s [14,15]. SLNs are sub-micron particles made from biocompatible lipids that are solid at room and body temperature [14]. When compared with liposomes, SLNs have a slower degradation rate *in vivo*, providing better control of drug release and superior protection of the incorporated drug [16]. Furthermore, these systems are easily and cost-effectively produced on a large scale by high pressure homogenization techniques (HPH) already available in the pharmaceutical industry for the production of

* Corresponding author. FarmaTec, Laboratório de Nanotecnologia Farmacêutica e Sistemas de Liberação de Fármacos, Faculdade de Farmácia, Universidade Federal de Goiás, Praça Universitária c/1a Avenida, Qd. 62. 74605-220 Goiânia, GO, Brazil. Tel.: +55 62 3209 6039; fax: +55 62 3209 6037.
E-mail addresses: rnmarreto@gmail.com, ricardomarreto@farmacia.ufg.br (R.N. Marreto).

0939-6411/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2011.02.012

parenteral O/W emulsions [14]. Another important preparation procedure for SLNs is the microemulsion method developed by Gasco [17]. This method utilizes a basic microemulsion protocol followed by the transfer of the warm microemulsion into cold water, resulting in the precipitation of the lipid phase forming solid nanoparticles [14]. Dilution of the microemulsion and HPH techniques are considered to be the most feasible methods for industrial production of SLNs [18].

Nanostrutured lipid carriers (NLCs) are an improved generation of SLNs produced by the controlled mixing of solid lipids with liquid lipids [19]. These particles display improved drug incorporation and release [19]. The advantages associated with using SLN and NLC for cancer chemotherapy were recently reviewed by Wong et al. [13].

Cytotoxic drugs of the camptothecin class have been encapsulated in lipid-based formulations [2]. The parent compound, camptothecin, is easily incorporated in solid lipid systems [20,21], because it is an extremely water-insoluble molecule. In contrast, TPT, like other hydrophilic compounds, does not partition well in melted lipid droplets during SLN/NLC preparation, representing a technological challenge for the development of these systems, because lipid nanoparticles of hydrophilic or ionic drugs will likely have poor entrapment and drug loading efficiency [13].

An interesting example of a formulation applied to solve this challenge of encapsulating hydrophilic compounds in SLN was reported by Liu et al. [22]. The authors successfully encapsulated the polypeptide insulin in an SLN by incorporating the drug in reverse micelles loaded in the lipid matrix using a double emulsion method. Development of new formulations and/or preparation methods remains a very important approach to improve the entrapment and drug loading efficiency of hydrophilic drugs in lipid nanoparticles [23]. Therefore, the overall aim of this work was to develop SLN and NLC formulations loaded with topotecan hydrochloride using a microemulsion technique, as an attempt to obtain nanoparticles of this clinically relevant camptothecin drug with high drug loading efficiency, prolonged release profile, increased chemical stability and cytotoxicity.

2. Materials and methods

2.1. Materials

Stearic acid (mp 58.77 °C, Vetec, Brazil) and oleic acid (Sigma–Aldrich, USA) were used as solid and liquid lipids for the preparation of SLNs and NLCs, respectively. Soy lecithin was purchased from Lipoid (Lipoid S100, Germany), and sodium taurodeoxycholate was purchased from Sigma–Aldrich (USA). TPT was obtained from Ricera Importation (Brazil). HPLC grade acetonitrile and methanol were purchased from JTBaker (USA). Sodium dihydrogen phosphate, sodium hydroxide, and acetic acid were obtained from Merck (Germany).

2.2. Methods

2.2.1. Production of SLN and NLC using a microemulsion technique

TPT loaded SLNs or NLCs were prepared using a microemulsion technique [24]. A mixture of lecithin, taurodeoxycholate, stearic acid, or stearic acid plus oleic acid was heated, and 250 μL of distilled water was added under magnetic stirring. A transparent, thermodynamically stable microemulsion system was formed. TPT was added to the microemulsion under slow mixing. This hot microemulsion was then dispersed into cold water (2–4 °C) under vigorous stirring (13,400 rpm for 10 min, IKA T25 Ultra-Turrax, Germany) using a 1:20 ratio (microemulsion:water) to form an SLN or NLC dispersion. The dispersions had a total lipid content of 2% (w/v), stabilized by 1.25% (w/v) of surfactant (lecithin:taurodeoxycholate, 4:1 ratio). The stearic acid:oleic acid ratio in the NLC preparation varied from 3:1 to 1:1. The amount of TPT and the temperature of the microemulsion varied are displayed in Table 1.

2.2.2. Mean particle size, polydispersity index (PdI) and zeta potential

Dynamic light scattering was used to assess the mean particle size and PdI of the SLN/NLC dispersions. Samples were diluted (1:100) with distilled water and analyzed using a Zetasizer Nano S (Malvern Instruments, UK). Zeta potential was determined from electrophoretic mobility using a Zetasizer Nano ZS (Malvern Instruments, UK).

2.2.3. Determination of drug entrapment efficiency (EE), drug loading (DL), and drug recovery (DR) for NLCs and SLNs

Quantitative determination of TPT was conducted by HPLC as previously described [25], using a Varian ProStar separation module equipped with a ProStar 310 UV detector, ProStar 240 solvent delivery pump and a ProStar 410 auto injector system (Varian Inc., USA). Chromatographic separation was achieved at 50 °C using a CromSep SS OminiSher 3 column (100 mm × 3.0 mm, 3 μm). The mobile phase was an 88:12 (v/v) mixture of acetate buffer-triethylamine (pH 5.5) and acetonitrile. The flow rate was 0.7 mL/min, the detection wavelength was 381 nm, and the injection volume was 10 μL. Validation of the method was performed according to international guidelines [26].

In order to isolate the entrapped TPT from free drug, 1 mL of freshly prepared SLN or NLC dispersions were subjected to ultrafiltration (Vivaspin 2®, Sartorius, USA) for 15 min at 4000*g* at 4 °C. Filtrate containing the free drug was withdrawn for HPLC analysis as described above. In order to validate the ultrafiltration system, drug retention was evaluated by subjecting an aqueous solution of free TPT to ultrafiltration followed by TPT quantification of the filtrate. All analyses were performed in triplicate.

Total amount of drug incorporated in the SLN/ NLC was determined by lipid disruption with acidified methanol followed by HPLC quantification. The entrapment efficiency (EE), drug loading (DL), and drug recovery (DR) were determined using the following equations:

$$\text{EE}\ (\%,\ \text{w/w}) = \frac{\text{amount of TPT in the formulation} - \text{amount of free drug}}{\text{amount of TPT in the formulation}} \times 100 \quad (1)$$

$$\text{DL}\ (\%,\ \text{w/w}) = \frac{\text{amount of TPT in the formulation} - \text{amount of free drug}}{\text{weight of the lipids(s)}} \times 100 \quad (2)$$

$$\text{DR}\ (\%,\ \text{w/w}) = \frac{\text{amount of TPT in the formulation}}{\text{amount of TPT added}} \times 100 \quad (3)$$

Table 1
Nanoparticle composition and microemulsion temperature.

Formulation	% TPT[a]	Oleic acid:stearic acid ratio	Temperature range (°C)
SLN1	4	–	100–125
SLN2	4	–	50–70
SLN3	8	–	50–70
NLC1	4	1:1	50–70
NLC2	4	1:3	50–70
NLC3	8	1:3	50–70
NLC4	10	1:3	50–70

[a] As a function of lipid mass.

2.2.4. In vitro drug release

Drug release was evaluated using a Franz-type diffusion cell supplied by Hanson Research (USA). A regenerated cellulose membrane with a molecular weight cut-off of 12,000 Da was used. The receptor compartment was filled with acetate buffer (pH 4.5), stirred at 300 rpm, and thermostated at 37 °C. Two hundred microliters of NLC or SLN dispersions was placed on the cellulose membrane in the donor compartment. Each experiment was run for 24 h under sink conditions ($n = 6$). At predetermined intervals, aliquots (500 μL) of receptor solution were withdrawn and replaced with fresh acetate buffer. Samples were diluted with acidified methanol and analyzed for TPT content by HPLC as described in Section 2.2.3. Drug release kinetics were determined by applying three kinetics models to the data to find the best fitting equations:

$$F = k_0 t \quad \text{Zero-order equation} \tag{4}$$

$$\ln F = \ln F - k_1 t \quad \text{First-order equation} \tag{5}$$

$$F = k_2 t_1/2 \quad \text{Higuchi equation} \tag{6}$$

where F represents the fraction of drug released over time t, and k_0, k_1, and k_2 are the apparent rate constants for zero-order, first-order, and Higuchi release constants, respectively.

2.2.5. Stability of TPT loaded in nanoparticles

To determine the effect of nanoparticle encapsulation on the stability of TPT, the degradation profile for free drug (aqueous solution, pH 4.5 and 7.4) and nanoparticle formulations (pH 7.4) were evaluated. Samples were incubated under orbital stirring (125 rpm) for 96 h at 25 or 37 °C. At time intervals of 0, 24, 48, and 96 h, aliquots were diluted with acidified methanol and analyzed for TPT content by HPLC.

2.2.6. Mass spectrometry analysis

Mass spectrometry analysis was performed using a 1200L Quadrupole MS/MS and a Micromass Global Q-TOF Ultima (MALDI/CapLC-ESI Quadrupole Time of Flight Mass Spectrometer). All experiments were conducted with ESI in positive mode. The temperature of the sample cooler in the autosampler was 20 °C. Chromatographic separation was achieved using a ZORBAX Rapid Resolution High Definition (RRHD) SB-C18 (50 mm × 2.1 mm, 1.8 μm) column. The mobile phases used were A: Water, 10 mM ammonium formate, pH 4.3; B: Acetonitrile. The flow rate was 0.4 mL/min; the injection volume was 1 μL of either topotecan aqueous solution or nanoparticle formulations. The mobile phase elution was accomplished using a gradient (0 min, 0% B; 0.25 min, 0% B; 9 min, 25% B; 11 min, 25% B; Stop time: 11 min; Posttime: 5 min)

2.2.7. Cytotoxicity assay

K-562 cells were cultured in RPMI 1640 medium (Gibco BRL Co., USA), containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37 °C. The growth and viability of K-562 cells were evaluated using the trypan blue exclusion test. Cells in culture were seeded in 96-well, flat, microtiter plates (1×10^6 cells/well) and incubated with free topotecan (50 μM, corresponding to the topotecan IC_{50}, according to Li et al. [27]) or an equivalent concentration of TPT loaded in either NLCs or SLNs. After 2 and 24 h incubation, 180 μL of trypan blue solution (0.2% in phosphate-buffered saline) was added to each well and the number of cells was estimated using a hemocytometer. Cells stained were scored as dead.

Cytotoxicity was evaluated by three separate experiments. Results were transformed into percentage of the control and expressed as the mean ± SD of three replicates.

2.2.8. Freeze-drying/re-dispersion of nanoparticles

Nanoparticles suspended in an aqueous trehalose solution (10–40%, w/v) were frozen at −20 °C overnight in glass vials. The freeze-drying process was carried out at −40 °C in a Micromodulyo-115, (ThermoFisher Scientific, England) at a pressure of 2.0 Pa for 8 h. Suspensions were reconstituted in ultrapurified water under gentle agitation. The mean diameter and PdI were evaluated.

2.2.9. Stability studies of freeze-dried nanoparticles

Selected freeze-dried nanoparticles were subjected to stability testing for 30 days in a desiccator at room temperature. Suspensions were reconstituted in ultrapurified water under gentle agitation, and the mean diameter, PdI, EE, and DR were evaluated.

2.2.10. Statistical analysis

Graph Pad Prism for Windows was used for statistical analysis. The statistical significance of each experiment was determined using the Student's t-test. Tests yielding p values <0.05 were considered significant.

3. Results and discussion

3.1. Validation of analytical procedures

The HPLC method for quantitative determination of topotecan hydrochloride was validated for linearity ($r^2 = 0.999$; $y = 19.87x + 6.67$), accuracy (97.53–103.14%), reproducibility (coefficient of variation, CV, <2.78%), and intermediate precision (CV < 3.40%). The HPLC method was selective, and the topotecan detection limit was 0.109 μg/mL with a quantitation limit of 0.365 μg/mL.

3.2. Formulation development of TPT-SLN and TPT-NLC

TPT-NLCs and TPT-SLNs prepared in this work using the microemulsion technique show high entrapment and drug loading efficiency values. The main properties of the resulting SLNs and NLCs are presented in Table 2. The average diameter and PdI values ranged from 108 to 168 nm and from 0.190 to 0.284, respectively. You et al. [28] and Ying et al. [29] found no significant effect of oleic acid on SLN particle size, when oil was incorporated at low concentrations (up to 10%, w/w). In contrast, Huang et al. [21] observed a considerable decrease in the average diameter of NLC containing squalene (50%, w/w), when compared with SLN. NLCs loaded with topotecan were prepared with oleic acid concentrations ranging from 25 to 50% (w/w); however, no effect on the average diameter and PdI due to liquid lipids was observed, even under higher oil concentrations. It is likely that these results, in contrast to those of Huang et al. [21], are due to differences in the preparation procedure and surfactant system employed.

A decrease in diameter of NLCs ($p < 0.05$) was observed when increasing amounts of topotecan were incorporated into formulations NLC 2, NLC 3, and NLC 4, which can be attributed to a co-surfactant effect of topotecan. In spite of a smaller diameter observed for NLC 4, data were not reproducible, probably due to the higher powder content in the formulation that altered the main properties of the formulation, including the drug loading efficiency. The effect of topotecan on average diameter of SLNs was not statistically significant.

In addition, all formulations studied showed EE values greater than 90% (Table 2), which was not improved by mixing oleic acid into the solid lipid matrix. Ruckmani et al. [30] reported encapsulation of hydrophilic methotrexate in solid lipid nanoparticles by a microemulsion technique and showed EE values of 51.52%. Another work on encapsulation of hydrophilic compounds into SLNs was reported by Marquele-Oliveira et al. [24], and the entrapment

Table 2
Mean diameter, polydispersity index (PDI), entrapment efficiency (EE), drug loading (DL), and drug recovery (DR) for TPT-SLN and TPT-NLC.

	Mean diameter (nm)	PdI	EE (%, w/w)	DL (%, w/w)	DR (%, w/w)
SLN1	162.05 (±3.18)	0.190 (±0.04)	91.79 (±0.16)	1.83 (±0,15)	49.74 (±4.01)
SLN2	165.10 (±3.25)	0.240 (±0.04)	91.80 (±0.24)	2.86 (±0,22)	77.81 (±5.44)
SLN3	159.30 (±5.44)	0.247 (±0.03)	94.75 (±1.13)	5.51 (±0,30)	72.80 (±2.37)
NLC1	152.37 (±7.89)	0.227 (±0.01)	95.13 (±1.08)	1.78 (±0,33)	46.84 (±8.17)
NLC2	168.76 (±1.72)	0.219 (±0.01)	93.20 (±0.40)	3.10 (±0,25)	77.62 (±6.21)
NLC3	143.52 (±3.74)	0.215 (±0.01)	96.82 (±1.42)	6.21 (±0,12)	80.26 (±1.53)
NLC4	108.28 (±23.47)	0.284 (±0.11)	92.18 (±4.05)	4.42 (±0,34)	47.96 (±9.15)

efficiency for this nitrosyl-ruthenium complex was 78.32%. It is important to note that in these cited literature reports, the lipid and surfactant compositions were very similar to those employed in this work. These differences, then, may be explained in terms of differences in drug solubility in melted lipids and the amount of drug added to the formulations. The high EE values observed in this study indicate that the lipid and surfactant compositions employed are very adequate for topotecan entrapment in solid lipid nanoparticles.

Drug loading capacity is a very relevant parameter for judging the suitability of a particular drug-carrier system [30]. In this study, DL values reached 6.21% (w/w) (see Table 2), which were suitable for the purpose of this work. The daily oral dose of topotecan is only 4 mg [1], and the TPT concentrations in the SLNs or NLCs developed in this work are high enough to produce solid or liquid dosage forms with acceptable weight and dimensions.

The NLC formulation with the highest oil concentration (CLN 1, Tables 1 and 2) showed low TPT recovery due to process losses, caused by the effect of oil on the stickiness of the lipid matrix, resulting in increased formulation adherence to the experimental apparatus.

Low TPT recovery was also observed in SLN1, which was prepared at a higher temperature (Tables 1 and 2). This can be attributed to TPT chemical degradation during the production of the nanoparticles. Previous work by Kearney et al. [31] shows the negative influence of temperature on TPT chemical stability. During SLN or NLC production, it is necessary to mix the drug into a previously melted lipid matrix. The level of thermal exposure of the drug depends on the preparation conditions and formulation parameters (type of lipid, type, and concentration of surfactant). In the present study, the influence of the preparation temperature on TPT stability was evaluated by the use of different temperature levels during the production of the nanoparticles. TPT recovery from SLN1 (100–125 °C) was significantly lower than from SLN2 (50–70 °C) (Table 2, $p < 0.01$). TPT chemical modifications during SLN production were confirmed by mass spectrometry. To identify newly emerging degradation products, a sample from the SLN1 formulation (formulation batch A) was taken and compared to a standard of pure topotecan drug compound. Both samples were injected five times to acquire accurate mass data and to obtain statistical data from the software-assisted data analysis. The obtained data files were grouped accordingly, for differential analysis with the XCMS profiling software [32,33]. For differential analysis of both groups, the molecular features (grouped molecular masses of isotopes and adducts belonging to a single compound at a particular retention time) of each group were displayed on a $\log_2$ abundance plot, showing the abundance ratio of the topotecan sample from formulation batch A against the topotecan standard sample (Fig. 1A).

In the plot, there are five lines corresponding to selected levels of abundance differences in the two sample groups. Molecular features lying on the middle line (1×) are equal in both groups, and the molecular features within the 2× margins were present at up to twofold abundance in one group and within the 4× margins up to fourfold. Beyond these margins, a feature was nearly unique or exclusively present in one group. The plot clearly shows a few molecular features with higher abundance in the degraded sample. Examination of the molecular feature data points showed molecular masses at $M = 364.3501$, $M = 348.1084$, and $M = 784.232$, with respective calculated empirical formulas, $C_{20}H_{16}N_2O_5$, $C_{20}H_{16}N_2O_4$, and $C_{42}H_{36}N_6O_{10}$. The calculated empirical formulas were confirmed by low relative mass errors of 0.35, 0.72, and 2.18 ppm.

The empirical formulas belong to the known topotecan derivatives, topotecan dimer (2), 10-hydroxycamptothecin (3), and camptothecin (4). The degradation reaction of topotecan (1), which is responsible for the generation of impurities in the final drug formulation, begins with dealkylation of the amine moiety yielding the first topotecan dimer as an intermediate (2). This key intermediate structure then yields, by deamination, 10-hydroxycamptothecin (3) and a loss of a hydroxyl group results in camptothecin (4) (Fig. 1B).

The base peak chromatogram (BPC) between *m/z* 300–900 shows peaks for the protonated compounds at retention times of 8.9 min for compound (2), 6.1 min for camptothecin (3), and 5.0 min for 10-hydroxycamptothecin (4). The main peak for topotecan was found at a retention time of 7.1 min (Fig. 1B). From the MS total ion chromatogram (TIC), ion chromatograms (EIC) for compound (2), (3), and (4) were extracted and all structures could be elucidated with high accuracy. It is clear from the above discussion that temperature control is key parameter to produce high quality TPT-SLN or TPT-NLC by microemulsion.

We were able to achieve up to 6.21% (w/w) TPT incorporation in lipid nanoparticles by adequately controlling process temperature and formulation. As a next step, we attempted to increase the amount of TPT incorporation in NLCs, because this type of lipid nanoparticle in theory should have a better capacity for incorporating drugs than SLNs [19]. Formulations using a higher concentration of TPT (NLC 4, Tables 1 and 2) were prepared. However, addition of higher amounts of powdered TPT caused rapid cooling of the microemulsion, which increased formulation adherence to the experimental apparatus and decreased drug yield. Therefore, the use of thermostatized devices is advisable for the transfer procedure, as proposed by Marengo et al. [34], to avoid process losses during industrial production of lipid nanoparticles. Due to the higher EE and DL values, NLC3 and SLN3 formulations were selected for further characterization.

3.3. Zeta potential and physical stability of freeze-dried SLN3 and NLC3

Table 3 shows zeta potentials for blank and TPT loaded-SLN3 and NLC3. All nanoparticles showed a high negative residual charge due to the chemical nature of the lipid matrix (stearic acid/oleic acid) and surfactants used. The incorporation of TPT decreased the zeta potential values ($p < 0.01$) for both SLN and NLC, because topotecan presents a positive residual charge at pH values used for the nanoparticles dispersions (4.2–4.6). No significant difference between the zeta potentials for NLC and SLN was observed.

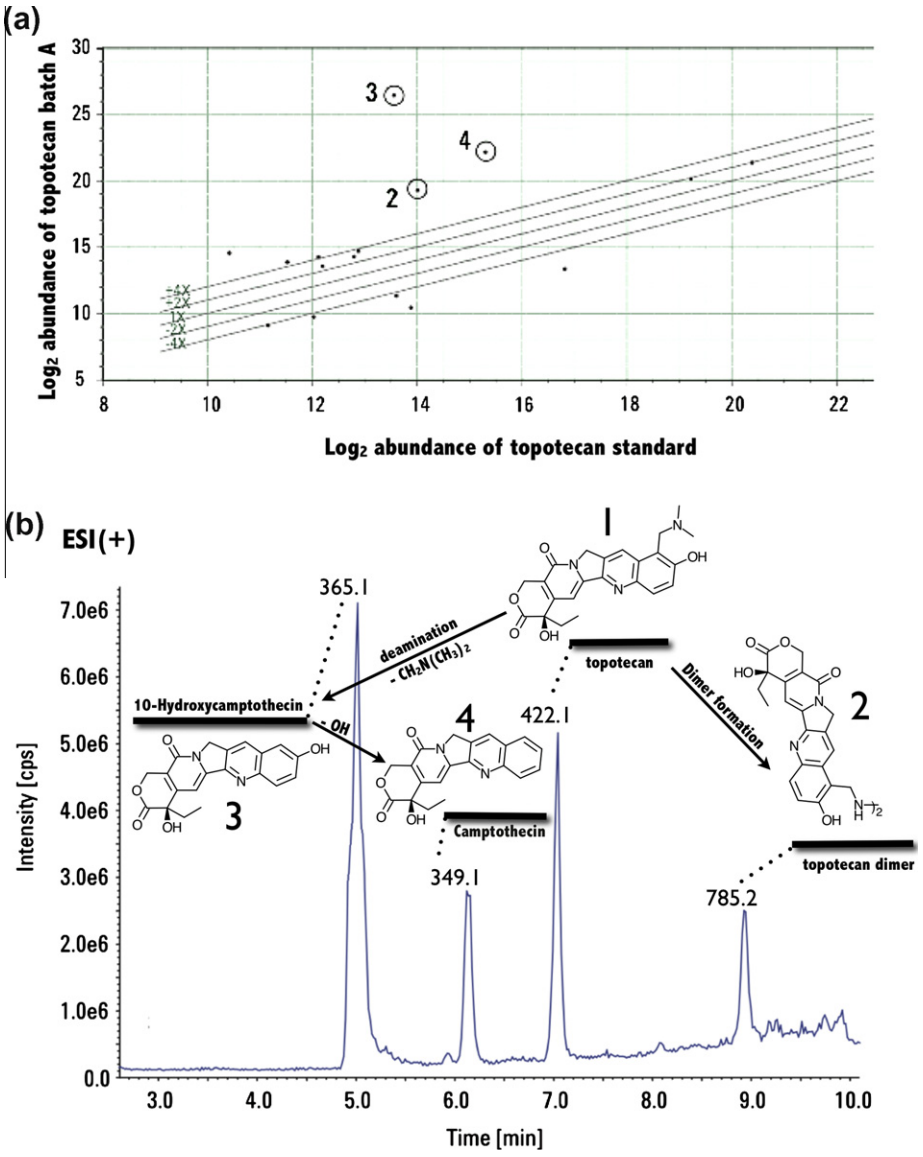

Fig. 1. Degradation reactions and products of topotecan. (1A) Comparison of a formulation batch of topotecan (SLN1) containing degradation products with a topotecan standard using XCMS analyte profiling software. (1B) Base peak chromatogram (BPC) of the degraded topotecan sample between m/z 300–900. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Zeta potential of blank and topotecan loaded lipid nanoparticles.

Formulation	Zeta potential
Blank SLN3	−53.86 (±0.30)
SLN3	−47.26 (±0.18)
Blank NLC3	−55.03 (±0.18)
NLC3	−45.31 (±1.29)

In order to freeze-dry SLN3 and NLC3, trehalose was used as cryoprotectant, and the influence of carbohydrate concentration was investigated. The presence of 15% (w/v) trehalose resulted in increased re-dispersion of the nanoparticles (Table 4). There were no significant differences between the average diameter and PdI of freshly prepared nanoparticles and freeze-dried, reconstituted nanoparticles. More concentrated trehalose solutions did not show any additional benefits.

Freeze-dried SLN3 and NLC3 dispersions containing 15% trehalose were also evaluated for stability. Fig. 2 shows the average diameter and PdI values for reconstituted nanoparticles 1, 10, and 30 days after lyophilization. There were no significant differences among the samples. In addition, entrapment efficiency and TPT recovery data were similar for all of the samples (Fig. 3).

3.4. TPT chemical stability in aqueous solution and in nanoparticle dispersions

In aqueous solution at pH 4.5, TPT remained stable for 96 h at 25 and 37 °C (Fig. 4). In contrast, TPT stability in aqueous media at pH 7.4 was lower and extremely temperature dependent (Fig. 5A and B). At 25 °C, about 20% of the TPT was degraded after 96 h for both free TPT, and NLC3, and SLN3 ($p < 0.01$) entrapped TPT (Fig. 5A), whereas at 37 °C, more than 80% of the TPT was degraded after 96 h. However, both lipid nanoparticles protected TPT against degradation ($p < 0.001$) (Fig. 5B). This is in agreement with the findings of Kearney et al. [31], which showed that TPT stability decreases with increased temperature and pH (in the acidic range). It should be noted that the initial TPT concentration in the

Table 4
Average diameter and PDI for freshly prepared and freeze-dried, reconstituted SLNs and NLCs.

Formulation	Average diameter (nm)		PdI	
	Before	After	Before	After
SLN3	159.30 (±5.44)	160.45 (±7.60)	0.247 (±0.030)	0.366 (±0.023)
NLC3	143.52 (±3.74)	139.3 (±1.20)	0.215 (±0.010)	0.228 (±0.014)

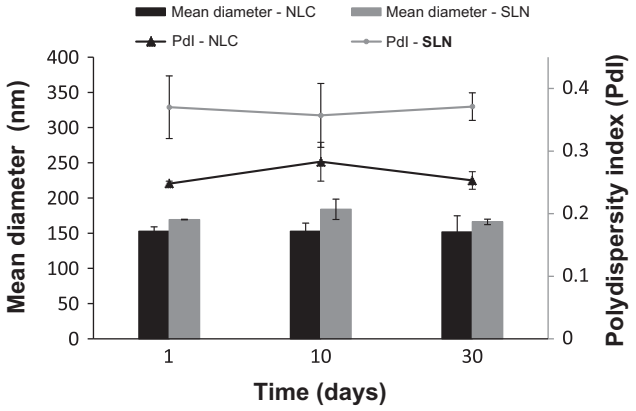

Fig. 2. Average size and PdI of reconstituted, freeze-dried nanoparticles after 1, 10, and 30 days of storage.

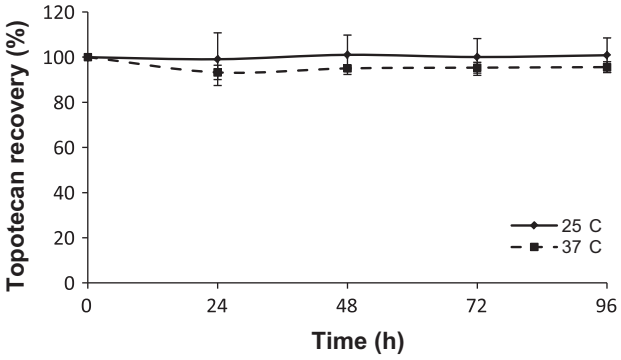

Fig. 4. Topotecan stability in aqueous solution (pH 4.5) at 25 and 37 °C.

nanoparticles was maintained after 24 h of exposure to pH 7.4 and 37 °C, indicating that *in vivo* TPT loaded nanoparticles should display increased stability and better performance compared with the free drug.

Camptothecins, such as topotecan, are S-phase-specific drugs, requiring a prolonged release profile for tumor cells [12]. Some researchers have evaluated TPT delivery by liposomes [11,35,36]. Laloo et al. [36] showed very rapid TPT release from liposomes, with 80% release after 60 min, whereas PEGylated and conventional liposomes obtained by Vali et al. [11] display prolonged TPT release with 50% and 68% of the drug released over a period of 10 h, respectively.

The release profiles of TPT from NLC3 and SLN3 are shown in Fig. 6. Diffusion of free TPT through the cellulose membrane was fast, and in 4 h reached approximately 75%. Statistical analysis showed differences in the diffusion of free drug and release of TPT-NLC and TPT-SLN after up to 12 h of experiment ($p < 0.001$). NLC and SLN sustained similar levels of TPT release for 12 h. Data from the first 12 h of the experiment were fitted to Higuchi, first-order, and zero-order equations. Release data from all samples best fit a Higuchi model ($r = 0.9957$ and 0.9938 for NLC and SLN, respectively). The calculated Higuchi release constants were 34.56 and 41.44 for NLC and SLN, respectively. There was a statistical difference between both nanoparticles and the free drug ($p < 0.001$), but there was no difference related to the presence of oleic acid in SLNs. Sustained TPT release from NLCs and SLNs may result in prolonged exposure of tumor cells to this drug, increasing clinical efficacy.

3.5. TPT cytotoxicity in different formulations

The cytotoxic effects of free TPT, TPT-NLC3, or TPT-SLN3 against K-562 cells were investigated, after 2 and 24 h of treatment, using the trypan blue exclusion method (Fig. 7).

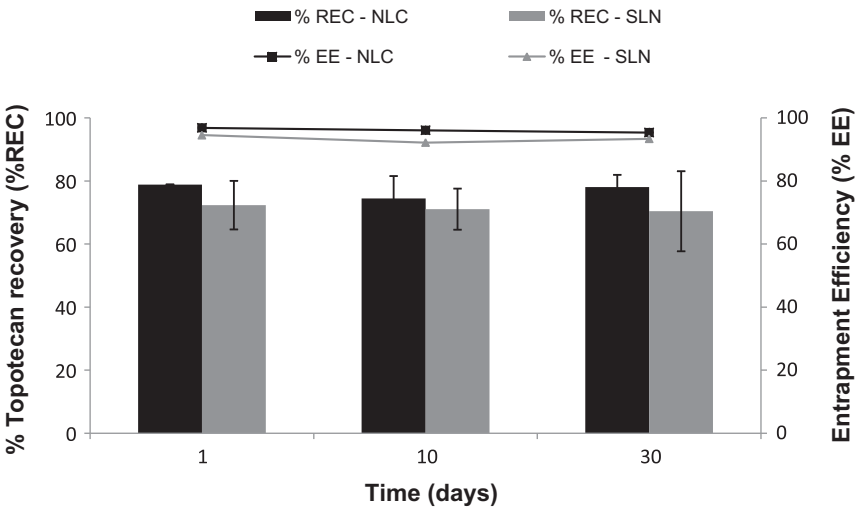

Fig. 3. Topotecan recovery and entrapment efficiency of reconstituted, freeze-dried nanoparticles after 1, 10, and 30 days of storage.

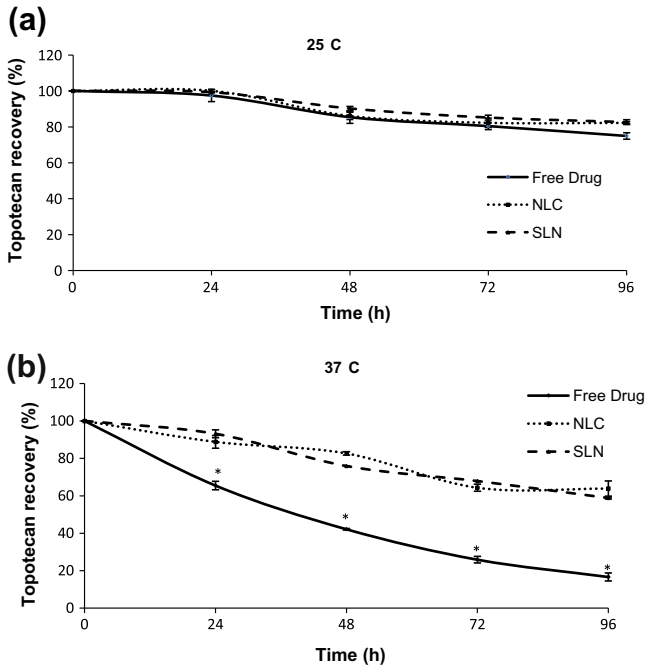

Fig. 5. Topotecan stability in aqueous solution (pH 7.4) and NLC/SLN dispersion (pH 7.4) at 25 °C (A) and 37 °C (B). $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

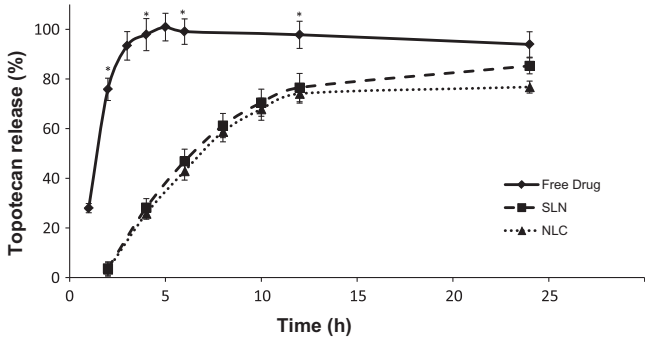

Fig. 6. Topotecan release from NLCs and SLNs. $^{*}p < 0.05$.

For all experiments, K-562 cells were exposed to the IC_{50} value of TPT, previously determined for leukemic cells by Li et al. [27]. As expected, exposure of K-562 cells to free TPT resulted in a significant decline in cell viability over both periods of investigation. Moreover, nanoencapsulation of TPT significantly enhanced this cytotoxic effect. After 2 h of K-562 incubation with NLC3 or SLN3, we observed a 90% enhancement of this cytotoxic effect for both nanoparticles when compared with the free drug. At 24 h, the increase in cytotoxicity was of 133.52% and 143.88% for SLN3 and NLC3, respectively. In this regard, Drummond et al. [12] demonstrated that TPT in liposome encapsulated form displayed improved cell uptake under physiological conditions and, probably as a consequence, increased cytotoxic effects. As mentioned above, physiological pH strongly induces the hydrolytic conversion of the TPT lactone active form to the inactive carboxylate form [10,37]. This conversion can be prevented by protection of the lactone moiety from aqueous environment [6]. Therefore, in this study, the improved TPT cytotoxicity against K-562 cells observed using SLN or NLC nanoparticles can be attributed to both an increase in cellular uptake of TPT and protection of the TPT lactone form. In support of this, *in vitro* release data (Fig. 6) showed that after 2 h of experiment, TPT was not released from lipid nanoparticles, which may contribute to the increased exposure of K-562 cells to the lactone TPT form. After 24 h of experiment, TPT was completely released from nanoparticles to the cell media. Thus, at this time point, the differences in cell viability are more likely related to drug internalization and subsequent release into the acidic environment of the cytoplasmic organelles involved in the phagocytosis process.

4. Conclusions

In this study, we report for the first time incorporation of a hydrophilic drug, TPT, into solid lipid nanoparticles. SLNs and NLCs were prepared using a microemulsion technique and displayed small, homogeneous size, high entrapment efficiency, and satisfactory drug loading. Nanoencapsulation sustained TPT release and improved its chemical stability and cytotoxicity. In spite of their different characteristics, there appear to be no differences between the NLCs and SLNs with respect to all of the parameters studied here. Both types of nanoparticles can be prepared by microemulsion, because precise control of processing temperature and drug exposure time to melted lipid can be established.

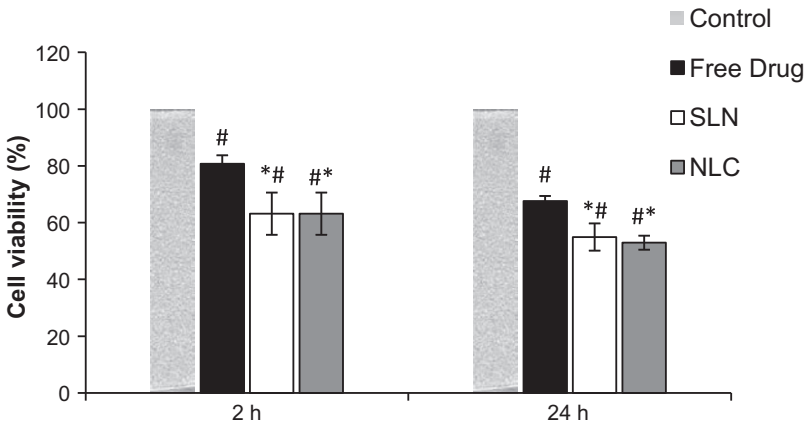

Fig. 7. Cytotoxicity of free and nanoencapsulated topotecan, incubated for 2 and 24 h with K-562 cells. $^{\#}p < 0.05$ referred to control; $^{*}p < 0.05$ referred to free drug.

Acknowledgment

The work was supported by Conselho Nacional de Pesquisa e Desenvolvimento (CNPq-MCT-Brasil), process number 577475/2008-8.

References

[1] C.J.H. Gerrits, H. Burris, J.H.M. Schellens, A.S.T. Planting, M.E.L. Van Den Burg, G.I. Rodrigez, V. Van Beurden, W.J. Loos, I. Hudson, S. Fields, J. Verweij, D.D. Von Hoff, Five days of oral topotecan (Hycamtin®), a phase I and pharmacological study in adult patients with solid tumours, Eur. J. Cancer 34 (1998) 1030–1035.
[2] A. Hatefi, B. Amsden, Camptothecin delivery methods, Pharm. Res. 19 (2002) 1389–1399.
[3] J.F. Pizzolato, L.B. Saltz, The camptothecins, Lancet 361 (2003) 2235–2242.
[4] P. Tardi, E. Choice, D. Masin, T. Redelmeier, M. Bally, T.D. Madden, Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models, Cancer Res. 60 (2000) 3389–3393.
[5] J. Fassberg, V.J. Stella, A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues, J. Pharm. Sci. 81 (1992) 676–684.
[6] T.G. Burke, A.K. Mishra, M.C. Wani, M.E. Wall, Lipid bilayer partitioning and stability of camptothecin drugs, Biochemistry 32 (1993) 5352–5364.
[7] T.G. Burke, X. Gao, Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine, J. Pharm. Sci. 83 (1994) 967–969.
[8] D. Subramanian, M.T. Muller, Liposomal encapsulation increases the activity of the topoisomerase I inhibitor topotecan, Oncol. Res. 7 (1995) 461–469.
[9] J.J. Liu, R.L. Hong, W.F. Cheng, K. Hong, F.H. Chang, Y.L. Tseng, Simple efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation, Anti-cancer Drugs 13 (2002) 709–717.
[10] Y.-L. Hao, Y.-L. Deng, Y. Chen, K.-Z. Wang, A.-J. Hao, Y. Zhang, In vitro cytotoxicity, in vivo biodistribution and anti-tumour effect of PEGylated liposomal topotecan, J. Pharm. Pharmacol. 57 (2005) 1279–1287.
[11] A.M. Vali, T. Toliyat, B. Shafaghi, S. Dadashzadeh, Preparation, optimization, and characterization of topotecan loaded PEGylated liposomes using factorial design, Drug Dev. Ind. Pharm. 34 (2008) 10–23.
[12] D.C. Drumond, C.O. Noble, Z. Guo, M.E. Hayes, C. Connolly-Ingram, B.S. Gabriel, B. Hann, B. Liu, J.W. Park, K. Hong, C.C. Benz, J.D. Marks, D.B. Kirpotin, Development of a highly stable and targetable nanoliposomal formulation of topotecan, J. Control. Release 141 (2010) 13–21.
[13] H.L. Wong, R. Bendayan, A.M. Rauth, Y. Li, X.Y. Wu, Chemotherapy with anticancer drugs encapsulated in solid lipid nanoparticles, Adv. Drug Deliv. Rev. 59 (2007) 491–504.
[14] R.H. Müller, K. Mader, S. Gohla, Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art, Eur. J. Pharm. Biopharm. 50 (2000) 161–177.
[15] W. Mehnert, K. Mader, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2001) 165–196.
[16] K. Vivek, H. Reddy, S.R.R. Murthy, Investigations of the effect of the lipid matrix on drug entrapment, in vitro release, and physical stability of olanzapine-loaded solid lipid nanoparticles, AAPS Pharm. Sci. Tech. 8 (2007) E1–E9.
[17] M.R. Gasco, Method of producing solid lipid microspheres having narrow size distribution, US Patent 5250,236, 1993.
[18] M. Muchow, P. Maincent, R.H. Muller, Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery, Drug Dev. Ind. Pharm. 34 (2008) 1394–1405.
[19] R.H. Muller, M. Radtke, S.A. Wissing, Nanostructured lipid matrices for improved encapsulation of drugs, Int. J. Pharm. 242 (2002) 121–128.
[20] S. Yang, J. Zhu, Y. Lu, B. Liang, C. Yang, Body distribution of camptothecin solid lipid nanoparticles after oral administration, Pharm. Res. 16 (1999) 751–757.
[21] Z.-R. Huang, S.-C. Hua, Y.-L. Yang, J.-Y. Fang, Development and evaluation of lipid nanoparticles for camptothecin delivery: a comparison of solid lipid nanoparticles, nanostructured lipid carriers, and lipid emulsion, Acta Pharmacol. Sin. 29 (2008) 1094–1102.
[22] J. Liu, T. Gong, C. Wang, Z. Zhong, Z. Zhang, Solid lipid nanoparticles loaded with insulin by sodium cholate-phosphatidylcholine-based mixed micelles: preparation and characterization, Int. J. Pharm. 340 (2007) 153–162.
[23] H. Yuan, S.-P. Jiang, Y.-Z. Du, J. Miao, X.-G. Zhang, F.-Q. Fu-Qiang Hu, Strategic approaches for improving entrapment of hydrophilic peptide drugs by lipid nanoparticles, Colloids Surf. B: Biointerfaces 70 (2009) 248–253.
[24] F. Marquele-Oliveira, D.C.A. Santana, S.F. Taveira, D.M. Vermeulen, A.R.M. Oliveira, R.S. Silva, R.F.V. Lopez, Development of nitrosyl ruthenium complex-loaded lipid carriers for topical administration: improvement in skin stability and nitric oxide release by visible light irradiation, J. Pharm. Biomed. Anal. 53 (2010) 843–851.
[25] D.L. Warner, T.G. Burke, Simple and versatile high-performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of camptothecin anticancer drugs, J. Chromatogr. B 691 (1997) 161–171.
[26] International Conference of Harmonization, Q2 (R1) Validation of Analytical Procedures: Text and Methodology, 2005.
[27] X. Li, W.L. Lu, G.W. Liang, G.R. Ruan, H.Y. Hong, C. Long, Y.T. Zhang, Y. Liu, J.C. Wang, X. Zhang, Q. Zhang, Effect of stealthy liposomal topotecano plus amlodipine on the multidrug-resistant leukaemia cells in vitro and xenograft in mice, Eur. J. Clin. Investig. 36 (2006) 409–418.
[28] J. You, F. Wan, F. Cui, Y. Sun, Y.-Z. Du, F.Q. Hu, Preparation and characteristic of vinorelbine bitartrate-loaded solid lipid nanoparticles, Int. J. Pharm. 434 (2007) 270–276.
[29] X.-Y. Ying, Y.-Z. Du, W.-W. Chen, H. Yuan, F.-Q. Hu, Preparation and characterization of modified lipid nanoparticles for doxorubicin controlled release, Pharmazie 63 (2008) 862–878.
[30] K. Ruckmani, M. Sivakumar, P.A. Ganeshkumar, Methotrexate loaded solid lipid nanoparticles (SLN) for effective treatment of carcinoma, J. Nanosci. Nanotechnol. 6 (2006) 1–5.
[31] A.S. Kearney, K. Patel, N.R. Palepu, Preformulation studies to aid in the development of a ready-to-use injectable solution of the antitumor agent, topotecan, Int. J. Pharm. 127 (1996) 229–237.
[32] H.P. Benton, D.M. Wong, S.A. Trauger, G. Siuzdak, XCMS2: processing tandem mass spectrometry data for metabolite identification and structural characterization, Anal. Chem. 80 (2008) 6382–6389.
[33] C.A. Smith, E.J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification, Anal. Chem. 78 (2006) 779–787.
[34] E. Marengo, R. Cavalli, O. Caputo, L. Rodriguez, M.R. Gasco, Scale-up of preparation process of solid lipid nanospheres, Part I, Int. J. Pharm. 205 (2000) 3–13.
[35] S.A. Abraham, K. Edwards, G. Karlsson, N. Hudon, L.D. Mayer, M.B. Bally, An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan with liposomes, J. Control. Release 96 (2004) 449–461.
[36] A. Laloo, P. Chao, P. Hu, S. Stein, P.J. Sinko, Pharmacokinetic and pharmacodynamic evaluation of a novel in situ forming poly(ethylene glycol)-based hydrogel for the controlled delivery of the camptothecins, J. Control. Release 112 (2006) 333–342.
[37] B.E. Davies, E.A. Minthorn, M.J. Dennis, H. Rosing, J.H. Beijnen, The pharmacokinetics of topotecano and its carboxylate form following separate intravenous administration to the dog, Pharm. Res. 14 (1997) 1461–1465.