Oral delivery of camptothecin using cyclodextrin/poly(anhydride) nanoparticles

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Abstract
Camptothecin (CPT), a molecule that shows powerful anticancer activity, is still not used in clinic due to its high hydrophobicity and poor active form's stability. In order to solve these drawbacks, the combination between poly(anhydride) nanoparticles and cyclodextrins was evaluated.
CPT-loaded nanoparticles, prepared in the presence of 2-hydroxypropyl-β-cyclodextrin, (HPCD-NP) displayed a mean size close to 170 nm and a payload of 50 µg per mg (25 times higher than the one of the control nanoparticles). CPT was not released from nanoparticles under gastric conditions. However, under intestinal conditions, about 50% of the drug content was released as a burst, whereas the remained drug was released following a zero-order kinetic. Pharmacokinetic studies revealed that the CPT plasma levels, from orally administered nanoparticles, were high and sustained up to 48 h. The CPT oral bioavailability was 7-fold higher than the value obtained with the control, whereas its clearance was significantly lower than for the aqueous suspension. These observations may be directly related to a prolonged residence time of nanoparticles in close contact with the intestinal epithelium, the presence of the cyclodextrin that decreases the CPT transformation into its inactive form and the generation of an acidic microenvironment during the degradation of the poly(anhydride) that would prevent the transformation of the active lactone into the inactive carboxylate conformation.

Key words: camptothecin, nanoparticles, cyclodextrins, oral delivery

Chemical compounds studied in this article
Camptothecin (PubChem CID: 24360); Gantrez AN (PubChem CID: 62708); 2-hydroxypropyl-β-cyclodextrin (PubChem CID: 44134771); β-cyclodextrin (PubChem CID: 444041); methyl-β-cyclodextrin (PubChem CID: 51051622).

1. Introduction

Oral chemotherapy is an attractive approach for cancer treatment because of its convenience, safety and patient acceptance, especially in chronic regimens [Borner et al., 2001; O'Neill and Twelves, 2002]. In recent studies, patients valued positively and expressed their preference for the oral route of administration since it interferes less with their daily life, giving them a feeling of freedom and a better quality of life [Halfdanarson and Jatoi, 2010]. Furthermore, oral administration avoids the discomfort of injection and can be eventually conducted at home. Unfortunately, for a number of anticancer drugs, their oral administration remains a challenge. This fact would be directly related to inadequate physico-chemical and stability properties as well as to the physiological barriers, which dramatically hamper the adequate absorption of such drugs.

20(S)-camptothecin (CPT) is a cytotoxic quinoline alkaloid which possess an affinity to the DNA enzyme topoisomerase I. The activity of CPT would be mediated by its binding to this enzyme during S-phase, leading to the accumulation of single DNA strands, and thus, to cell death [Li et al., 2006]. Nevertheless its therapeutic application was (and remains to be) hindered by a very low solubility in aqueous media, high toxicity, and rapid inactivation through lactone ring hydrolysis at physiological pH conditions. In fact, CPT exists in a dynamic equilibrium between the closed-ring lactone moiety and the open-ring carboxylic acid form (Figure 1). The first form (lactone) is responsible for the molecule's powerful anticancer activity, whereas the carboxylate form shows a reduced efficacy associated with a high toxicity [Lorence and Nessler, 2004]. These two forms coexist at 50% at a pH of 6.65, being the equilibrium moved towards the lactone form at lower pH and favoring the carboxylate open ring at higher pH [Wall et al., 1966]. Regrettably, the open-ring form, predominant at physiological pH shows less than 10% potency of the closed-ring form as topoisomerase I inhibitor [Sriram et al., 2005]. This fact would be related to the high affinity of the carboxylate form for human serum albumin, making it inaccessible for cellular uptake [Sun et al., 2012].

In order to solve these drawbacks, CPT derivatives, such as topotecan and irinotecan were synthesized. Both were approved for clinical use in Japan, Europe and USA [Oberlies and Kroll, 2004]. Although they offer the advantage of their better water solubility, camptothecin still shows lower IC50 against a variety of cancer cell lines [Thomas et al., 2004]. More recently, other approaches based on the use of drug delivery systems have been also proposed including nanoparticles [Min et al., 2008], liposomes [Watanabe et al., 2008], micelles [Kawano et al., 2006], polymer derivatives [Weiss et al., 2013], and solid lipid nanoparticles [Yang et al., 1999; Martins et al., 2013].

Another possible strategy would be the incorporation of CPT in poly(anhydride) nanoparticles made from the copolymer of methylvinylether and maleic anhydride. In the recent past, these nanoparticles have demonstrated an important capability to develop adhesive interactions with the gut epithelium and improve the oral bioavailability of different drugs such as fluorouridine [Arbos et al., 2004], paclitaxel [Agüeros et al., 2010; Zabaleta et al., 2012] and atovaquone [Calvo et al., 2011]. In addition, these nanoparticles can be easily combined with different ligands and excipients in order to improve their mucus-permeating properties and/or to modify their distribution within the gastrointestinal tract [Arbos et al., 2002; Calleja et al., 2014]. In this particular

work, the combination of these poly(anhydride) nanoparticles with cyclodextrins has been studied. This combination may offer interesting advantages. First, the use of cyclodextrins facilitates the encapsulation of lipophilic drugs in polymeric nanoparticles increasing the resulting payload [Agüeros et al., 2010; Calvo et al., 2011]. Second, the presence of cyclodextrins modulates and sustains the release of lipophilic compounds from polymeric nanoparticles [Agüeros et al., 2009; Penalva et al., 2015]. Third, in the particular case of camptothecin, cyclodextrins may improve up to 10 times the lactone form's half-life [Kang et al., 2002]. In addition, some cyclodextrins (i.e., 2-hydroxypropyl-β-cyclodextrin) display an inherent ability to inhibit different enzymatic complexes and extrusion pumps localized in the intestinal epithelium [Ishikawa et al., 2005; Zhang et al., 2011].

Therefore, the aim of this work was to optimize the preparative process of camptothecin-loaded poly(anhydride) nanoparticles combined with cyclodextrins as well as to evaluate the in vitro properties and in vivo capabilities of the developed nanoparticles to promote the oral absorption and bioavailability of this drug in Wistar rats.

2. Materials and methods

2.1. Reagents

Poly(methyl vinyl ether-co-maleic anhydride) or poly(anhydride) (PMV/MA) [Gantrez® AN 119; MW 200,000] was purchased from ISP (Barcelona, Spain). Camptothecin (CPT) (99.0%) was supplied by 21CECpharm (London, UK). β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), pepsin and pancreatin were obtained from Sigma Aldrich (Germany) whereas methyl-β-cyclodextrin (M-β-CD) and sulfopropyl-β-cyclodextrin (SP-β-CD) were from Cyclolab (Hungary). Acetone, ethanol, acetonitrile and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Deionized reagent water was prepared by a water purification system (Wasserlab, Spain). All reagents and chemicals used were of analytical grade.

2.2. Solubility studies

CPT solubility studies were carried out according to the Higuchi and Connors method [Higuchi and Connors, 1965]. An excess of CPT was added to a deionised aqueous solution in vials containing increasing amounts of the oligosaccharide (β-CD, HP-β-CD, M-β-CD or SP-β-CD). These flasks were sonicated for 5 minutes, sealed and shaken in a VorTemp 56^{TM} Shaking Incubator (Labnet International Inc., USA) at 25°C for 72 hours. Then, samples were filtered (0.45 μm) and the concentration of CPT was determined by HPLC (see Section 2.4.2). The presence of trace amounts of cyclodextrins did not interfere with the assay. The assays were performed in triplicate.

The apparent stability constants (K_c) and the stoichiometry of the camptothecin-cyclodextrin complexes (CPT-CD) were estimated from the phase solubility diagrams. For A_L diagrams, the apparent stability constant (K_c) of the drug-oligosaccharide complex can be calculated as follows [Loftsson et al., 2004]:

$$K_c = \frac{\text{slope}}{S_0\,(1\text{-slope})} \qquad [\text{Eq.1}]$$

where S_0 is the molar solubility of camptothecin in absence of cyclodextrins and the slope is obtained from the initial straight-line portion of the plot of camptothecin concentration against the cyclodextrin concentration.

Moreover, the complexation efficiency (CE) was calculated from the slope of the linear phase of the phase-solubility diagram [Loftsson et al., 2005]:

$$CE = S_0 K_c = \frac{slope}{1\text{-}slope} \qquad [Eq.2]$$

2.3 Preparation of CPT-loaded nanoparticles

Nanoparticles were prepared following a previous protocol [Calvo et al., 2011] with minor modifications. In brief, 100 mg poly(anhydride) were firstly dissolved in 5 mL acetone. Then, variable amounts of camptothecin and cyclodextrins were dispersed in this solution and the mixture was incubated at room temperature for variable time. Nanoparticles were formed by the addition of ethanol and water (1:1 v/v). The organic solvents were eliminated under reduced pressure (Büchi R210, Switzerland) and the resulting suspensions were filtered through a 0.45 µm membrane. Nanoparticles were purified twice by centrifugation at 27,000 x g, for 20 min in a 3K30 centrifuge (Sigma Centrifuges, UK). Supernatants were removed and pellets resuspended in water. Finally, the formulations were frozen and freeze-dried (Genesis 12EL, Virtis, USA) using sucrose (5% w/w) as cryoprotector. Control nanoparticles were prepared in the same way but in the absence of the oligosaccharide.
The developed formulations were named as follows: SPCD-NP (CPT-loaded nanoparticles containing SP-β-CD), HPCD-NP (CPT-loaded nanoparticles containing HP-β-CD), MCD-NP (CPT-loaded nanoparticles containing M-β-CD and NP (CPT-loaded nanoparticles in absence of oligosaccharide).

2.4 Characterization of the CPT nanoparticles

2.4.1 Physicochemical characterization

The mean hydrodinamic diameter of the nanoparticles and the zeta potential were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a ZetaPlus analyzer system (Brookhaven Instruments Corporation, New York, USA). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25ºC by dynamic light scattering angle of 90ºC. The zeta potential was determined as follows: 200 µL of the same samples were diluted in 2 mL of a 0.1 mM KCl solution. The morphology of the nanoparticles was examined by scanning electron microscopy (SEM) in a Zeiss DSM940 digital scanning electron microscope (Oberkochen, Germany) coupled with a digital image system (Point Electronic GmBh, Germany). The yield of the process was calculated by gravimetry as described previously [Arbos et al., 2002].

2.4.2 Camptothecin content in nanoparticles

The amount of camptothecin loaded into nanoparticles was quantified by HPLC. Briefly, the equipment was an Agilent model 1100 series LC and fluorescence detector set at excitation and emission λ of 380 and 418 nm, respectively. The chromatographic system was equipped with a reversed-phase 150 mm x 3 mm C18 Phenomenex Gemini column (particle size 5 µm) and precolumn (Phenomenex Security Guard C18). The mobile phase, pumped at 1 mL/min,

consisted on a mixture 50:50 (v/v) of acetonitrile and trifluoroacetic acid 0.01% (v/v). The column was placed at 30ºC and the injection volume was 20 µL. Calibration curves were designed over the range of 0.48 and 8000 ng/mL (R^2> 0.999). The limit of quantification was calculated to be 1.3 ng/mL with a relative standard deviation of 4.1%.
For analysis, nanoparticles were solubilized (1 mg/mL) with acetonitrile and further diluted in the same solvent (1:100 v/v). Samples were transferred into auto-sampler vials, capped and placed in the HPLC auto-sampler. Each sample was assayed in triplicate and results were expressed as the amount of camptothecin (in µg) per mg nanoparticles. The encapsulation efficiency (EE) was calculated as follows:

$$EE(\%) = (Q_{associated}/Q_{initial}) \times 100 \qquad [Eq.3]$$

where $Q_{initial}$ is the initial amount of CPT added and $Q_{associated}$ is the amount of entrapped CPT in the nanoparticles, which was calculated by HPLC.

2.5. *In vitro* release study

Release experiments were conducted at 37ºC using simulated gastric (SGF; pH 1.2; pepsin 0.32% w/v) and intestinal (SIF; pH 6.8; pancreatin 1% w/v) fluids. In order to ensure sink conditions, both media included polysorbate 80 (1% w/v). Studies were performed under agitation in a Vortemp 56^{TM} Shaking Incubator (Labnet International Inc., NJ, USA) after the dispersion of the nanoparticles in the appropriate medium.
For each time point, 2.4 µg CPT formulated in nanoparticles were resuspended in 1 mL of the corresponding simulated fluid. The different formulations were kept in the SGF for 2 hours. Then, samples of nanoparticles were centrifuged and transferred to vials and incubated for 18 hours in SIF. At different points, sample tubes were collected, transferred to Vivaspin tubes (300,000 MWCO, Sartorius group, Germany) and centrifuged at 3000xg for 5 minutes. The amount of CPT released from the formulations was quantified by HPLC (calibration curves of free CPT in supernatants obtained from SGF and SIF, R^2> 0.999).
For experiments in SIF, the data obtained after the initial burst step were fitted to a zero-order kinetic model (Eq. 4). This model has been suggested for drug delivery systems in which the matrix releases the same amount of drug by unit of time [Costa and Sousa Lobo, 2001]:

$$Q_t = Q_0 + K_{Z0}\, t \qquad [Eq.4]$$

Where Q_t is the amount of drug dissolved in time t and Q_0 is the initial amount of drug in the solution (most times, $Q_0 = 0$) and K_{ZO} is the zero-order release constant. We have used a modified zero-order equation, where we have replaced the Q_t and Q_0 term with Mt/M∞ (fraction of released drug at time t), and K_{Z0} is the zero order release constant.

2.6. *In vivo* pharmacokinetic studies in Wistar rats

Pharmacokinetic studies were performed in Wistar rats obtained from Harlan (Barcelona, Spain). Studies were conducted in accordance with the ethical guidelines and policies for investigations in laboratory animals approved by the Ethical Committee for Animal Experimentation of the University of Navarra

(protocol number 058-12) in accordance with the European legislation on animal experiments. Before the experiment, animals were adaptively fed for 1 week with free access to food and drinking water (22±2ºC; 12-h light and 12-h dark cycles; 50-60% relative humidity). Before the oral administration of the formulations, animals were fasted overnight to avoid interference with the absorption, allowing free access to water.

For the pharmacokinetic study, rats were randomly divided into 4 groups (n=6). The experimental groups, receiving a single oral dose of CPT, were as follows: (i) HPCD-NP 1 mg/kg, and (ii) HPCD-NP 5 mg/kg. For the administration, nanoparticles were dispersed in 1 mL water and the resulting suspension was administered orally to animals. As controls, 2 different groups of animals received a camptothecin suspension either intravenously (via tail vein) or orally. This camptothecin suspension was prepared extemporary by dispersing the drug (3.6 mg) in 10 mL of a solution of 10% Tween 80 and saline (9:1 by vol.) and sonicated for 20 minutes to breack up aggregates [Fox et al., 2009].

Blood samples were collected at set times after administration. EDTA was used as anticoagulant agent. Blood volume was recovered intraperitoneally with an equal volume of normal saline solution pre-heated at body temperature. Samples were immediately placed on ice and centrifuged at 2,500xg for 10 minutes. Plasma was separated into clean tubes and kept frozen at -20ºC until HPLC analysis.

2.6.1. Determination of CPT plasma concentration by HPLC

The amount of CPT was determined in plasma by HPLC and fluorescence detection with the technique described above (see Section 2.4.2). The only difference was that the injection volume was 100 µL. In addition, calibration curves and quality controls were designed over the range of 0.48 and 8000 ng/mL (R^2>0.999) by adding appropriate volumes of standard camptothecin in a mixture of dimethylsulfoxide, acetonitrile and trifluoroacetic acid 1:8.9:0.1 (by vol.) to drug free plasma.

For analysis, an aliquot (100 µL) of plasma was mixed with 400 µL of acetonitrile and vortexed for 2 min in order to achieve the precipitation of the plasma proteins. After centrifugation (5000xg, 5 min), the supernatant was collected and evaporated until dry (Savant, Barcelona, Spain). Finally, the residue was dissolved in 120 µL of reconstitution solution (dimethylsulfoxide, acetonitrile and trifluoroacetic acid; 1:8.9:0.1 v/v/v) and transferred to autosampler vials, capped and placed in the HPLC autosampler. The limit of quantification was calculated to be 2.6 ng/mL with a relative standard deviation of 4.6%.

2.6.2. Pharmacokinetic data analysis

The pharmacokinetic analysis of plasma concentration plotted against time data, obtained after administration of the different CPT formulations, was performed using a non-compartmental model with the WinNonlin 5.2 software (Pharsight Corporation, USA). The following parameters were estimated: maximal plasma concentration (C_{max}), time in which C_{max} is reached (T_{max}), area under the concentration-time curve from time 0 to t (AUC), mean residence time (MRT), clearance (Cl), volume of distribution (V) and half-life in the terminal phase ($t_{1/2}$).

Furthermore, the relative oral bioavailability, Fr, of CPT was expressed as the ratio between the area under the concentration-time curve from time 0 to t (AUC) of the formulations assayed (HPCD-NP) and the one of the oral suspension of camptothecin administered.

2.7. Hematological studies

At the end of the pharmacokinetic study, and before the sacrifice of the animals, blood samples were collected in order to evaluate different hematological and biochemical values. The following hematological parameters were analyzed in a Sysmex XT1800i hematology analyzer (Roche Diagnostics Ltd., Switzerland): hemoglobin (HGB, g/L), hematocrit (HCT, %), red blood corpuscles count (RBC, 10^{12}/L), white blood corpuscles count (WBC, 10^{9}/L), absolute erythrocyte indices and white blood cells. On the other hand, biochemical analyses of plasma samples were performed with a Hitachi 911™ analyser (Roche Diagnostics, Switzerland), following the protocols standardized by the manufacturer. The following parameters were calculated: aspartate transaminase (AST, U/L), alanine transaminase (ALT U/L) and urea (mg/dL).

2.8. Statistical analysis

For the physicochemical and pharmaceutical characterization of formulations, data are expressed as the mean ± standard deviation (S.D.) of at least three experiments. For in vivo results, the non-parametric Kruskall-Wallis followed by Mann-Whitney U-test was used to investigate statistical differences. In all cases, P values lower than 0.05 were considered as statistically significant difference. All data processing was performed using GraphPad Prism 5.0 statistical software (GraphPad Software, USA).

3. Results

3.1 Solubility studies

The phase-solubility diagrams for the complex formation between camptothecin and the different cyclodextrins are presented in Figure 2. This plot shows that the solubility of the drug in water increased linearly as a function of the oligosaccharide concentration. For all the cyclodextrins tested, the solubility diagram of camptothecin can be classified as an A_L type [Higuchi and Connors, 1965]. These results would theoretically suggest the formation of a CPT-cyclodextrin complex with a probable 1:1 stoichiometry. The apparent stability constants, K_c, obtained from the slopes of the linear phase solubility diagrams (Table 1) were found to be of about 237 M^{-1} (for β-CD), 130 M^{-1} (for HP-β-CD), 93 M^{-1} (for SP-β-CD) and 453 M^{-1} (for M-β-CD).

The complexation efficiency was calculated to be 0.0024, 0.0014, 0.0011 and 0.0052 for β-CD, HP-β-CD, SP-β-CD and M-β-CD, indicating that on average, the water-soluble system formed by the oligosaccharide and camptothecin involves a molar ratio of CPT/CD of 1/400, 1/700, 1/900 and 1/200, respectively.

3.2. Optimization of the preparative process of camptothecin-loaded nanoparticles

As a first approach, bare poly(anhydride) nanoparticles (prepared in the absence of cyclodextrins) were evaluated in order to know their capability to load camptothecin. The resulting carriers displayed a mean size of about 130

nm and a payload of 2.2 µg CPT per mg nanoparticles. Thus, in order to improve this payload, nanoparticles were prepared by incorporating cyclodextrins. For the optimization of the preparative process of CPT-loaded poly(anhydride) nanoparticles in the presence of cyclodextrins, two parameters were studied: (i) incubation time between the three main components of the formulation (drug, cyclodextrin and polymer) before the formation of nanoparticles by desolvation and (ii) the CPT/oligosaccharide ratio. All the studies were performed with an initial bulk amount of polymer of 100 mg.

Figure 3 shows the influence of the incubation time in acetone between the different compounds forming the nanoparticles (HP-β-CD, CPT and the polymer) on the drug loading and the encapsulation efficiency of the resulting carriers. Surprisingly, the best results were obtained when nanoparticles were formed after just 1 minute of incubation between the components of the formulation (polymer, cyclodextrin and drug). Under these circumstances, the payload of the resulting nanoparticles was 50 µg CPT per mg nanoparticles. This amount represented 22-times more camptothecin than that observed for bare nanoparticles. However, longer times of incubation negatively influenced the CPT loading and the encapsulation efficiencies. Thus, 60 min of incubation between the three main components of the formulation lead to a drug loading of about 15 µg CPT/mg with an EE of 6%.

The next step was to evaluate the influence of the CPT/cyclodextrin molar ratio on the drug loading and the encapsulation efficiency. Figure 4 summarizes these results.

For a CPT/ HP-β-CD ratio of 1, the payload of the resulting nanoparticles was close to 50 µg/mg and similar to the value observed when a ratio of 2.4 was used (Figures 3 and 4). Nevertheless the encapsulation efficiency was significantly higher for a CPT/cyclodextrin ratio of 1 than when a ratio of 2.4 was used ($p>0.05$). When the CPT/oligosaccharide ratio was lower than 1, the CPT loading significantly decreased ($p>0.05$; Figure 4).

For sulphopropyl-β-cyclodextrin and methyl-β-cyclodextrin based nanoparticles, a similar influence of the incubation time and the CPT/oligosaccharide ratio was found (data not shown). In addition, for the three cyclodextrins tested, when the amount of oligosaccharide used to prepare the nanoparticles was higher than 18% of the polymer mass, the process resulted always in the aggregation of the nanoparticles.

3.3. Characterization of the CPT-loaded nanoparticles

Based on the former optimization results, nanoparticles containing cyclodextrins were prepared at a CPT/cyclodextrin molar ratio of 1 and with an incubation time (between the components of the formulation) of 1 minute. The physicochemical characteristics of the resulting poly(anhydride) nanoparticles containing CPT are summarized in Table 2. Overall, nanoparticles containing cyclodextrins displayed a mean size slightly higher than bare nanoparticles (150-170 nm vs 130 nm). Interestingly, in all cases, the polydispersity index (PDI) was lower than 0.2, which implies homogeneous formulations. Concerning the zeta potential, all nanoparticle formulations showed a negative surface charge; slightly lower for nanoparticles containing cyclodextrins than for control ones. On the other hand, the yield of the process significantly increased when cyclodextrins were incorporated in the preparative procedure of

nanoparticles. In addition, nanoparticles containing HP-β-CD displayed a slightly higher yield than when SP-β-CD or M-β-CD were used.
Finally, for nanoparticles containing cyclodextrins, the drug loading was between 17 and 25 times higher than for control nanoparticles (NP). Comparing the different cyclodextrin tested, nanoparticles containing HP-β-CD displayed the highest capability to encapsulate camptothecin into poly(anhydride) nanoparticles (around 50 µg/mg).
The morphological analysis by scanning electron microscopy (Figure 5) showed that nanoparticles containing cyclodextrins consisted of homogeneous population of spherical particles, with a smooth appearance and a mean size similar to that obtained by photon correlation spectroscopy.

3.4 In vitro release study.

CPT release kinetics from nanoparticles was evaluated in simulated gastric and intestinal fluids (containing polysorbate 80 as solubilizing agent for CPT). Figure 6 represents the release profiles of camptothecin from the different assayed formulations as a function of time. Overall, all the nanoparticle formulations displayed a similar release behaviour. This pattern was characterised by a first non-release step, when nanoparticles were dispersed in SGF, and a release step (when nanoparticles were dispersed in SIF), in which around 50% of the loaded drug was rapidly released followed by a more sustained deliverance phase. This second phase in SIF was characterized by a continuous release of campthotecin for at least 15 hours.
This release profile of camptothecin from nanoparticles containing cyclodextrins, after the initial burst effect, seemed to be linear and constant. Therefore zero order equation [Eq. 4] was fitted to the second step of the release curve in SIF. This sustained release of camptothecin from these nanoparticles was well adjusted to this model (Table 3) characterised by R^2 higher than 0.89 in all cases.

3.5. In vivo pharmacokinetic studies in Wistar rats

The plasma concentration-time profile of CPT after a single intravenous administration at a dose of 1 mg/kg is shown in Figure 7. Camptothecin was administered as a suspension with a particle size of 1500±144 nm and a PDI of 0.44±0.05. Data were analysed by a non-compartmental model. CPT plasma concentration decreased rapidly after the administration, not being detectable 6 hours after the administration. After administration, the drug plasma concentration reached 430 ng/mL (C_{max}) and the values for AUC and $t_{1/2z}$, were 0.39 µg h/mL and 0.69 hours, respectively. The CPT clearance and the volume of distribution were calculated to be 755 mL/h and 683 mL, respectively (Table 4).
Figure 8 shows the plasma concentration profiles of CPT after the administration of a single oral dose (1 mg/kg or 5 mg/kg) to rats as either a suspension or loaded in nanoparticles. When the drug suspension was administered orally, the plasma levels increased rapidly reaching the C_{max} 30 min after administration. Then, sustained plasma levels of the drug were maintained for at least 4 hours and, finally, the CPT levels decreased rapidly. Thus, 10 hours after administration, not detectable levels of CPT in plasma were observed.

For camptothecin-loaded in nanoparticles (HPCD-NP), and administered at the same dose (1 mg/kg), the main difference with the aqueous suspension formulation was that the drug levels were quantified in plasma for a more extended period of time. Thus, animals that received CPT included in nanoparticles showed a first phase characterized by increasing levels of drug in plasma for the first 0.5 to 6 hours, followed by a slow and prolonged decrease phase with quantifiable levels of CPT until 48 hours. This fact was confirmed when HPCD-NP was administered to animals at a dose of 5 mg/kg (Figure 8).
Table 4 summarises the main pharmacokinetic parameters estimated with a non-compartmental analysis of the experimental data obtained after the administration of the different formulations to rats. The drug suspension showed similar AUC values when administered intravenously or orally (0.39 and 0.38 µg h/mL, respectively) being the C_{max} 5 times higher for the intravenous route. The MRT was significantly higher when administered by the oral route than intravenously (1 hour versus 5.9 h; $p< 0.01$). Similarly, the volume of distribution was significantly higher when camptothecin was administered by the oral route than intravenously ($p< 0.05$). No statistical differences were observed for the clearance of camptothecin or for the half-life of the drug in the terminal phase of the curve.
For HPCD-NP, the camptothecin AUC was found to be about 6.5-times higher than when the drug was orally administered as a suspension. In a similar way, the MRT was also higher when CPT was administered in the form of nanoparticles (31 h vs 5.9 h; $p < 0.01$). It is noteworthy that for HPCD-NP, the clearance of camptothecin was significantly lower than for the drug suspension ($p< 0.05$). This fact would be due to an important improvement of $t_{1/2z}$. Besides, the relative oral bioavailability of camptothecin was found to be 6.9 fold higher when loaded in nanoparticles than when administered as oral suspension.
Finally, for HPCD-NP administered at a dose of 5 mg/kg, the pharmacokinetic parameters were found to be quite similar to those calculated at a 1 mg/kg dose. The main difference would be the decrease observed in the relative oral bioavailability (6.9 vs 3.3) after the application of the dose correction.

3.6. Hematological studies

Figure 9 shows some hematological and biochemical values of the animals at the end of the pharmacokinetic study. The administration of CPT produced a significant decrease in RBC and HGB compared to the control ($p<0.01$). Furthermore, a significant decrease in white blood cells was also observed for animals treated orally with CPT ($p<0.05$). Regarding the biochemical parameters, the levels of AST, ALT and urea nitrogen for animals treated with CPT were found to be similar to that of the control (non-treated) animals.

4. Discussion

Camptothecin is a highly lipophilic compound with a low oral bioavailability that shows a low stability related with a rapid hydrolysis of its lactone ring under physiological conditions ($t_{1/2}$ of approximately 22 min at 37ºC and pH 7.4) [Mi and Burke, 1994; Sun et al., 2012]. This reaction is a reversible pH-sensitive interconversion from the potent lactone form (stable below pH 5) to the poorly active, and even toxic, carboxylate form (stable above pH 8) (Figure 1) [Mi and Burke, 1994]. In an attempt to minimize these drawbacks, the aim of this work

was to evaluate the capability of poly(anhydride) nanoparticles for the oral delivery of camptothecin when associated to cyclodextrins.

In our experimental conditions, the phase solubility diagrams of camptothecin in presence of different cyclodextrins (Figure 2) can be considered as A_L type, suggesting that the complex is hydrosoluble and formed following a 1:1 stoichiometry. At a concentration of 1.5% w/v, β-CD (near its solubility limit in water) increased camptothecin's solubility 3 times compared to that of the free drug (0.011mM in water). Regarding the three modified cyclodextrins tested (HP-β-CD, M-β-CD and SP-β-CD), at a concentration of 20% w/v, they were able to enhance the drug's solubility in water by factors of 24, 67 and 22, respectively. These data are in accordance with those reported previously by other research groups that suggested that the significantly higher solubilizing effect of M-β-CD compared to other cyclodextrins would be attributed to the presence of methyl groups, which enlarge the whole cavity of the molecule by extending the secondary hydroxyl side and narrowing the primary hydroxyl side of the cone [Uekama, 1985; Kang et al., 2002]. On the other hand, the low complexation efficiency values calculated for all the CPT/cyclodextrin complexes suggested a tendency of cyclodextrins and their complexes to self-associate by forming aggregates in aqueous media [Messner et al., 2010]. This phenomenon would probably be promoted by the incubation time.

Nanoparticles were prepared by a simple desolvation method. The mean size of the camptothecin-loaded nanoparticles when prepared in the presence of cyclodextrins appeared to be slightly higher than those obtained by control nanoparticles. On the other hand, the incorporation of cyclodextrins resulted effective in improving the camptothecin payload; however, prolonging the incubation time between the different components of the formulation (oligosaccharide, drug and polymer) prior the formation of nanoparticles negatively affect the drug loading (Figure 3). This observation may be directly related to the formation of more stable CPT:cyclodextrin complexes, by increasing the incubation time. As a consequence, the migration of the drug:oligosaccharide complexes to the aqueous phase during the formation of nanoparticles (by the desolvation of the polymer with the addition of ethanol and water; section 2.3) would be facilitated. In any case, under the selected experimental conditions, the incorporation of cyclodextrins increased the camptothecin loading up to 25 times higher than when nanoparticles were prepared in the absence of these oligosaccharides (Table 2). Surprisingly, in spite of its modest ability to increase the aqueous solubility of the drug, HP-β-CD was found to be the most successful oligosaccharide to promote the camptothecin encapsulation in the poly(anhydride) nanoparticles, reaching a drug loading close to 50 µg per mg nanoparticle. When M-β-CD was used during the preparation of nanoparticles (M-NP), the camptothecin drug loading was about 15% lower than when HP-β-CD was used. This fact may be due to the higher affinity of CPT for M-β-CD that would drag the drug to the aqueous phase during the formation of nanoparticles by desolvation. In any case, the CPT loadings (between 3.4 and 5%, Table 2) were higher than those published previously by other authors including PLGA nanoparticles (2.6% CPT) [Cirpanli et al., 2009], poly(e-caprolactone) nanoparticles (1.4%) [Cirpanli et al., 2009], or poly(aspartic) nanoparticles (1.96%) [Zeng et al., 2013].

Concerning the in vitro release of CPT from these nanoparticles, it is noteworthy that under acidic conditions (SGF), no drug release was observed for any of the

formulations tested (Figure 6). However, when nanoparticles were dispersed in simulated intestinal fluid (neutral pH conditions), camptothecin was released. Under these SIF conditions, the release was characterized by a burst effect of about 50% of the drug content followed by continuous slow release of the remaining content of drug in the nanoparticles (Figure 6). This second step followed a zero-order kinetic (Table 3), in which CPT was released at a rate close to 2.5 %/h. All of these results may be directly related with the behavior of poly(anhydride) nanoparticles in different media. In fact, in an aqueous environment, the anhydride groups of the nanoparticles are hydrolyzed yielding two carboxylic acid residues (-COOH). When nanoparticles are in an acidic environment, these carboxylic acids are not ionized and the polymer would adopt a compact conformation, decreasing the porosity of the nanoparticles [Agüeros et al., 2009]. On the contrary, when the nanoparticles moved to a neutral aqueous medium, nanoparticles would swell due to an ionization of the carboxylic acids residues.

For the pharmacokinetic study, single doses of 1 and 5 mg/kg were selected. Amongst all the nanoparticle formulations developed, HPCD-NP was chosen for the pharmacokinetic study, since it was the most successful regarding CPT loading. The intravenous administration of a single dose of camptothecin as a suspension (1 mg/kg) to laboratory animals produced initial high drug levels in plasma, followed by a rapid biphasic decline over time (Figure 7). Six hours after the administration, the plasma drug levels were close to the quantitation limit of the analytical technique. This profile is in accordance with previous results described by other authors who suggested that this rapid decline of plasma drug levels could be due to an embolization or retention of the drug particulates in the lung capillaries [Martins et al., 2013].

When the same suspension was administered orally, the C_{max} was 5-times lower than the iv administration; although, plasma levels decreased more gently and CPT was quantified up to 8 h post-administration. The CPT clearance of the oral suspension was similar to that calculated when administered iv; however, the half-life of the terminal phase was about 2.5 fold higher than when administered intravenously ($p<0.05$). For HPCD-NP (1 mg/kg), the plasma levels of camptothecin were high and sustained until 48 h after administration. For this formulation, the relative bioavailability was calculated to be 6.9-times higher than for the drug oral suspension. This fact would be related to the capability of these nanoparticles to reach the gut mucosa, prolonging their residence in close contact with the intestinal epithelium, in which they would release their cargo and, hence, facilitating the camptothecin absorption in a continuous way [Calleja et al., 2014]. Interestingly, the clearance of camptothecin, when administered as HPCD-NP, was significantly lower than for the suspension ($p< 0.05$), whereas the half-life of the terminal phase was calculated to be about 15-times higher when CPT was loaded in nanoparticles than formulated as suspension ($p<0.01$). Different reasons may explain this behavior including the prolonged residence time of nanoparticles in the absorptive membrane, the presence of cyclodextrin molecules that prevent the formation of the CPT carboxylate open-ring form [Kang et al., 2002], and the generation of an acidic microenvironment due to the degradation of the polymeric nanoparticles via hydrolysis of the poly(anhydride). As a consequence, CPT would be protected from its transformation into the inactive

form till its complete release from nanoparticles, facilitating its absorption and arrival to the systemic circulation in the lactone conformation.
Regarding the administration of HPCD-NP at a dose of 5 mg/kg, pharmacokinetic parameters were similar to those found with the same formulation administered at a dose of 1 mg/kg. Nevertheless, the relative bioavailability (adjusted to the dose) was found to be 2-times lower than that calculated with the low dose (1 mg/kg). This observation would be related to the specific mechanisms involved in the intestinal absorption of camptothecin. In fact, CPT is absorbed via passive diffusion and carrier-mediated active processes [Gupta et al., 2000]. Therefore, it is plausible to think that with the high dose (5 mg/kg), the intestinal transporters involved in the absorption of CPT would be saturated. A similar phenomenon has been proposed for other lipophilic compounds such as paclitaxel and some platinum complexes [Mckeage et al., 1995; Malingré et al., 2001].
Finally, 48 hours after the anticancer drug administration, hematological and biochemical analyses were carried out. It has been reported that CPT and its derivatives can induce severe inhibition of marrow leading to the change of hematological parameters [Bülbül Hizel et al., 2008]. More particularly, CPT decreases red blood cells, white blood cells and platelet counts and hemoglobin [Singh and Marar, 2011; Luo et al., 2016]. The tested groups received the drug suspension (iv and oral, 1 mg/kg) and HPCD-NP (5 mg/kg). As shown in Figure 9, in all cases, CPT caused important decreases in RBC and HGB compared with the non-treated animals. Similarly, when CPT was administered orally (as suspension or in the form of nanoparticles), the white blood cells were also lower than for normal rats ($p<0.05$). Interestingly, the group of animals receiving the highest dose of CPT (HPCD-NP, 5 mg/kg) exhibited similar values than the group of animals treated with the intravenous suspension of the anticancer drug (1 mg/kg). On the other hand, ALT and ASM levels are usually used to monitor liver diseases [Jiang et al., 2011], whereas an increase in serum urea nitrogen is the marker for kidney damage [Singh and Marar, 2011]. In this case, none of the formulations tested induced significant changes in any of these biochemical parameters.
In summary, poly(anhydride) nanoparticles containing HP-β-CD were able to load a significant amount of camptothecin and provide gastrorresistant properties to the resulting formulation (HPCD-NP). After the oral administration of these nanoparticles to rats, the camptothecin plasma levels were high and sustained in time up to 2 days offering a relative oral bioavailability which was calculated to be at least 7 fold higher than that observed for the control. These plasma levels with other evidences (such as the decreased drug clearance) suggest that these nanoparticles would provide a long residence time in the intestinal mucosa and, at the same time, would be capable of protecting the transformation of the camptothecin lactone form to the carboxylate conformation that is facilitated at neutral pH conditions.

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Figure captions

Figure 1. Chemical structure of camptothecin in its lactone form and carboxylate conformation.

Figure 2. Phase solubility diagrams of CPT-cyclodextrin systems in purified water at 25ºC. Data show the amount of camptothecin solubilised as a function of the amount of cyclodextrin in the medium. β-CD: β-cyclodextrin; HP-β-CD: hydroxypropyl-β-cyclodextrin; SP-β-CD: sulfopropyl-β-cyclodextrin; M-β-CD: methyl-β-cyclodextrin. Data expressed as mean±SD, n=3.

Figure 3. Influence of the incubation time between the components of the formulation (polymer, cyclodextrin and drug in acetone) before the formation of nanoparticles on the camptothecin loading (bars, left axis) and encapsulation efficiency (line, right axis). NI: 1 min of incubation. Experimental conditions: CPT/cyclodextrin molar ratio of 2.4; polymer 100 mg. Data expressed as mean±SD, n=3.

Figure 4. Influence of the camptothecin/HP-β-CD ratio on both the drug loading (continuous line, left axis) and the encapsulation efficiency (dotted line, right axis) of the resulting nanoparticles. Experimental conditions: polymer 100 mg; no incubation time after the mixing of the three compounds in acetone (polymer, oligosaccharide and drug). Data expressed as mean±SD, n=3.

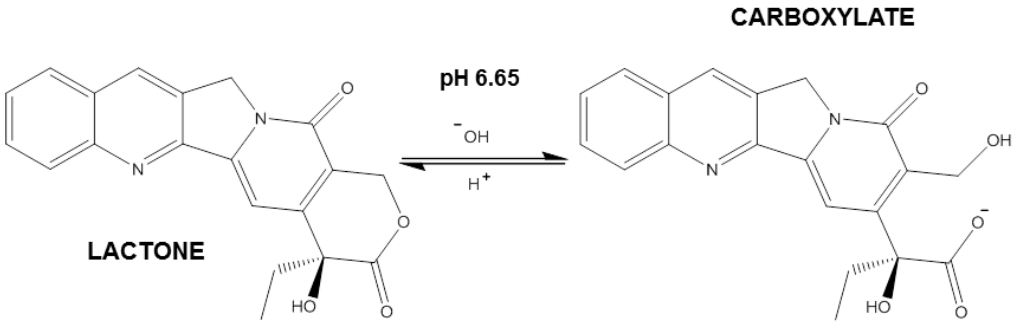
Figure 5. Scanning electron microscopy (SEM) of freeze-dried poly(anhydride) nanoparticles. SPCD-NP: CPT-loaded nanoparticles containing SP-β-CD; HPCD-NP: CPT-loaded nanoparticles containing HP-β-CD; MCD-NP: CPT-loaded nanoparticles containing M-β-CD.

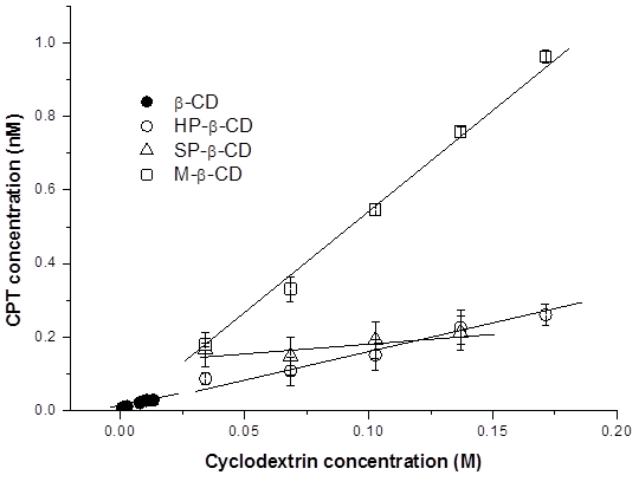
Figure 6. Camptothecin release profile from poly(anhydride) nanoparticles containing cyclodextrins (SPCD-NP, HPCD-NP, MCD-NP) after incubation in simulated gastric fluid (0-2 h) and simulated intestinal fluid (2-18 h) under sink conditions at 37ºC. Data expressed as mean±SD, n=3. SPCD-NP: CPT-loaded nanoparticles containing SP-β-CD; HPCD-NP: CPT-loaded nanoparticles containing HP-β-CD; MCD-NP: CPT-loaded nanoparticles containing M-β-CD.

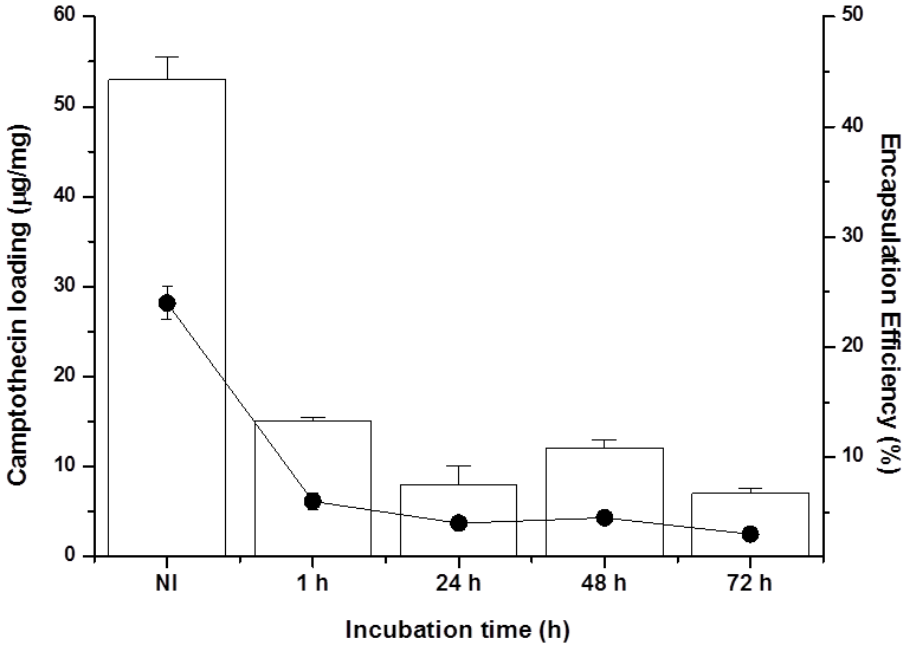
Figure 7. Concentration-time profile of CPT in male Wistar rats after a single intravenous dose of the drug formulated as aqueous suspension (1 mg/kg). Data expressed as mean (±SD), n=6.

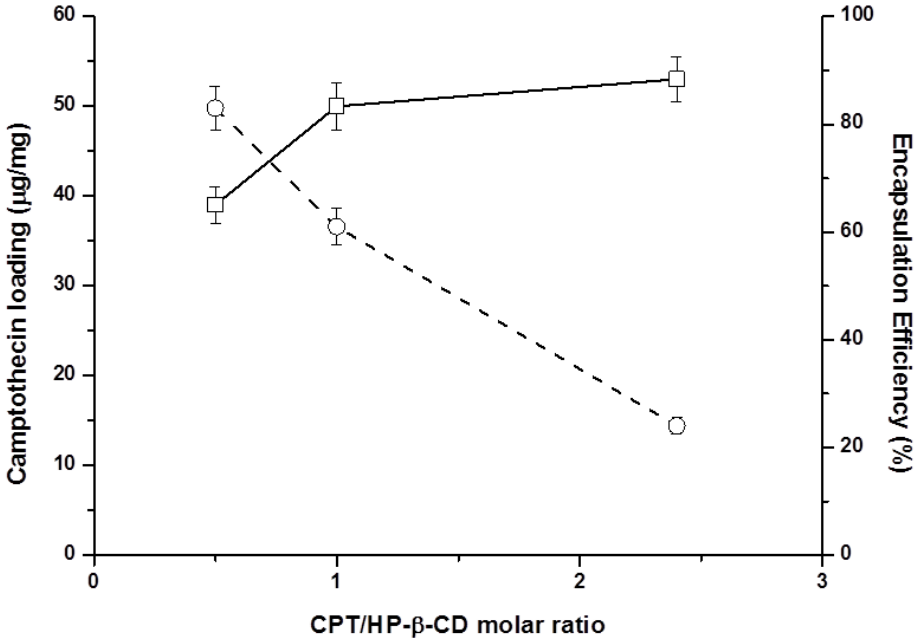
Figure 8. Concentration-time profile of CPT in male Wistar rats after a single oral dose of the suspension (1 mg/kg) or HPCD-NP (1 mg/kg or 5 mg/kg). Data expressed as mean (±SD), n=6.

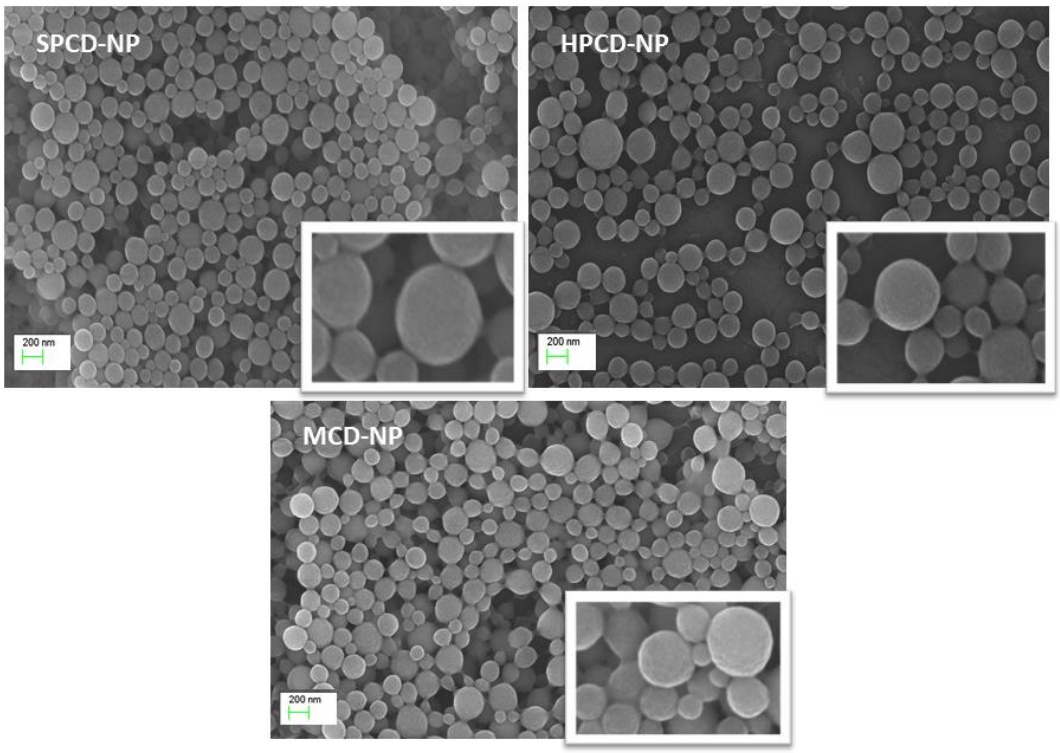
Figure 9. Hematological (A) and biochemical (B) parameters of blood in Wistar male rats 48 hours after administration of a single dose of camptothecin as suspension (intravenously, CPT iv 1 mg/kg, or nanoparticles (HPCD-NP 5 mg/kg). Data expressed as mean (±SD). (n=6). NR: normal rats; WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; AST: aspartate aminotransferase; ALT: alanine transaminase; Urea: urea nitrogen.

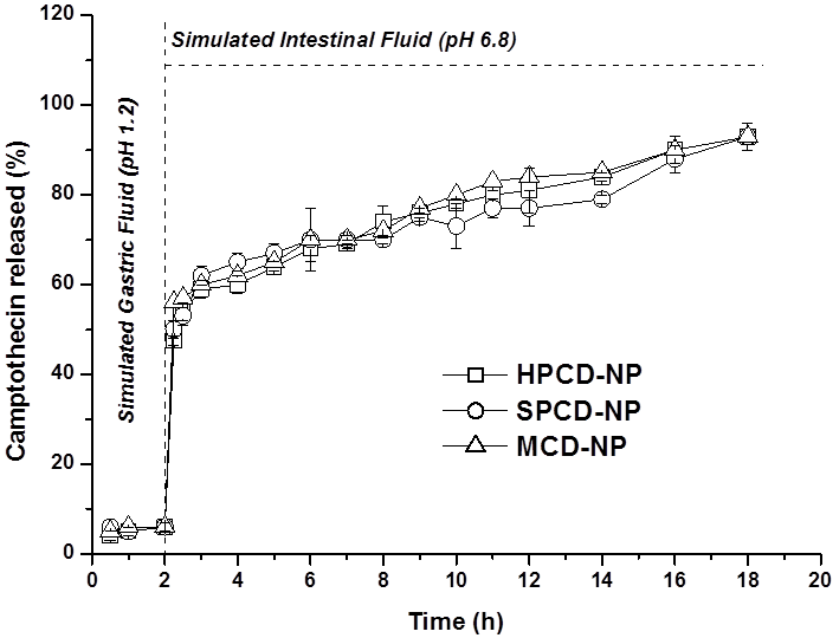

CARBOXYLATE
pH 6.65
$^-$OH
H^+
LACTONE
N
O
HO
OH
O$^-$

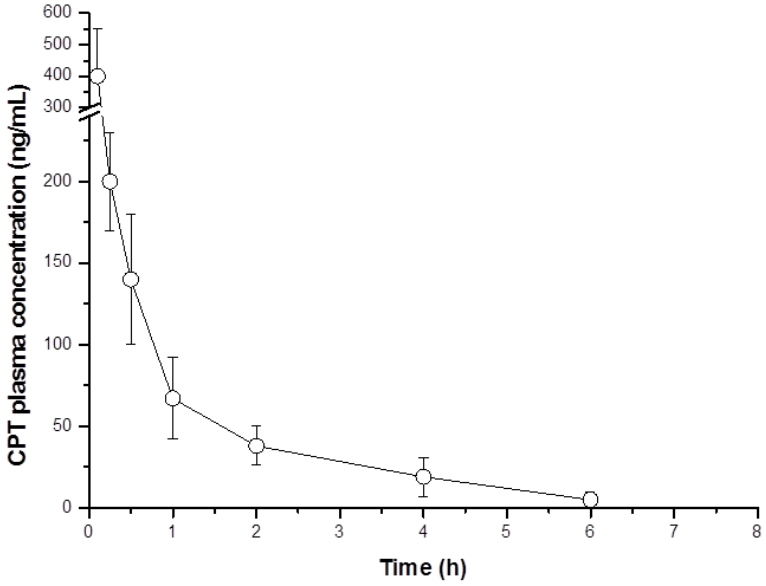

β-CD
HP-β-CD
SP-β-CD
M-β-CD
CPT concentration (nM)
Cyclodextrin concentration (M)
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.05
0.10
0.15
0.20

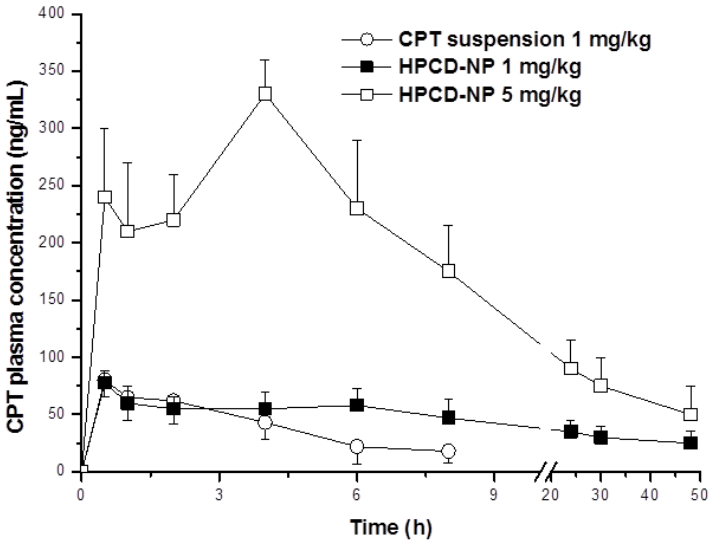

Camptothecin loading (µg/mg)
Encapsulation Efficiency (%)
Incubation time (h)
NI
1 h
24 h
48 h
72 h
0
10
20
30
40
50
60


Camptothecin loading (μg/mg)
Encapsulation Efficiency (%)
CPT/HP-β-CD molar ratio
0
10
20
30
40
50
60
0
20
40
60
80
100
0
1
2
3


SPCD-NP
200 nm
HPCD-NP
200 nm
MCD-NP
200 nm


Simulated Intestinal Fluid (pH 6.8)
Simulated Gastric Fluid (pH 1.2)
Camptothecin released (%)
Time (h)
HPCD-NP
SPCD-NP
MCD-NP
0
20
40
60
80
100
120
0
2
4
6
8
10
12
14
16
18
20


CPT plasma concentration (ng/mL)
600
500
400
300
200
150
100
50
0
0
1
2
3
4
5
6
7
8
Time (h)


CPT suspension 1 mg/kg
HPCD-NP 1 mg/kg
HPCD-NP 5 mg/kg
CPT plasma concentration (ng/mL)
Time (h)
400
350
300
250
200
150
100
50
0
0
3
6
9
20
30
40
50

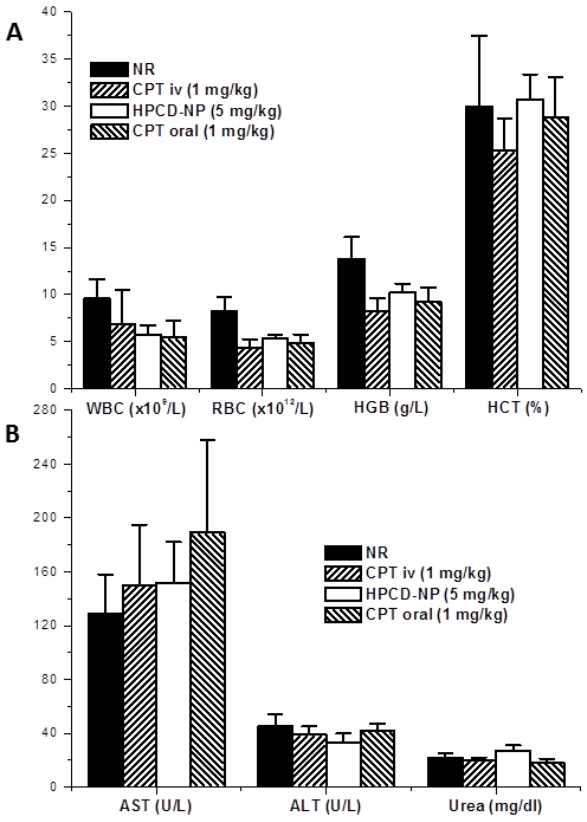

A
NR
CPT iv (1 mg/kg)
HPCD-NP (5 mg/kg)
CPT oral (1 mg/kg)
0
5
10
15
20
25
30
35
40
WBC (x10^{9}/L)
RBC (x10^{12}/L)
HGB (g/L)
HCT (%)
B
NR
CPT iv (1 mg/kg)
HPCD-NP (5 mg/kg)
CPT oral (1 mg/kg)
0
40
80
120
160
200
240
280
AST (U/L)
ALT (U/L)
Urea (mg/dl)

Table 1. Apparent stability constants (K_c), complexation efficiencies (CE) and maximal solubility at the highest concentration of the oligosaccharide for the complexation of CPT with different cyclodextrins in deionised water at 25ºC. β-CD: β-cyclodextrin; HP-β-CD: hydroxypropyl-β-cyclodextrin; SP-β-CD: sulfopropyl-β-cyclodextrin. M-β-CD: methyl-β-cyclodextrin. Data expressed as mean±SD, n=3.

Complex	**Slope ($x10^5$)**	**Kc (M^{-1})**	**Maximal CPT solubility (mM)**	**CE ($x10^5$)**
CPT / β-CD	273±2	237±18	0.03±0.01	241±5
CPT / HP-β-CD	151±2	130±20	0.26±0.03	143±2
CPT / SP-β-CD	114±1	96±15	0.20±0.04	110±2
CPT / M-β-CD	525±2	453±14	0.96±0.01	524±1

Table 2. Physico-chemical characterization of poly(anhydride) nanoparticles. NP: CPT-loaded nanoparticles in the absence of oligosaccharides; SPCD-NP: CPT-loaded nanoparticles containing SP-β-CD; HPCD-NP: CPT-loaded nanoparticles containing HP-β-CD; MCD-NP: CPT-loaded nanoparticles containing M-β-CD. Data expressed as mean±SD, n=5.

	Size (nm)	**PDI**	**Zeta potential (mV)**	**Yield (%)**	**CPT loading (µg/mg NP)**	**EE (%)**
NP	130±4	0.10±0.05	-45±2	36±5	2.2±0.3	2.6±0.8
SPCD-NP	157±4	0.18±0.01	-37±2	51±5	34±2.5	34.7±6.2
HPCD-NP	148±6	0.15±0.04	-40±1	61±6	50±2.6	61.0±9.5
MCD-NP	168±5	0.17±0.01	-44±1	58±4	43±3.5	49.9±7.8

Table 3. Analysis of the linear step of the camptothecin release from nanoparticles when incubated in simulated intestinal fluid (pH 6.8; pancreatin 1% w/v). SPCD-NP: CPT-loaded nanoparticles containing SP-β-CD; HPCD-NP: CPT-loaded nanoparticles containing HP-β-CD; MCD-NP: CPT-loaded nanoparticles containing M-β-CD.

	Zero-order		
	K_{Z0} (h^{-1})	**M_0 /M_∞ initial**	**R^2**
SP-NP	0.02±0.01	0.61±0.01	0.888
HP-NP	0.02±0.01	0.58±0.01	0.971
M-NP	0.02±0.01	0.59±0.01	0.960

Table 4. Pharmacokinetic parameters estimated after the intravenous or oral administration of a single dose of camptothecin (1 mg/kg or 5 mg/kg) formulated as suspension or encapsulated in nanoparticles. HPCD-NP: CPT-loaded nanoparticles containing hydroxypropyl-β-cyclodextrin. Data expressed as mean (±SD); (n=6).

	Dose (mg/kg)	Route	AUC (µg h/mL)	C_{max} (µg/mL)	T_{max} (h)	$t_{1/2z}$ (h)	Cl (mL/h)	V (mL)	MRT (h)	Fr
CPT suspension	1	iv	0.39±0.12	0.43±0.25	0.01	0.69±0.13	755±289	683±147*	1±0.4	-
CPT suspension	1	oral	0.38±0.21	0.08±0.02	0.5	1.6 ± 0.8	619±357	1545±414	5.9±1.4	1
HPCD-NP	1	oral	2.6±1.8*	0.08±0.01	1.5	20.1±6.1**	134±61*	2847±1038	31.0±7.1**	6.9
HPCD-NP	5	oral	6.2±3.1	0.35±0.05	3.5	16.6±5.5	161±73	3583±745	24.9±9.3	3.3

AUC: area under the concentration-time curve from time 0 to t h; C_{max}: peak plasma concentration; T_{max}: time to peak plasma concentration; $t_{1/2z}$: half-life of the terminal phase; Cl: clearance; V: volumen of distribution; MRT: mean residence time; Fr: relative oral bioavailability. *p<0.05, ** p<0.01, HPCD-NP 1 mg/kg vs. oral CPT suspension 1 mg/kg.