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Development and validation of a simple reversed-phase HPLC method for the determination of camptothecin in animal organs following administration in solid lipid nanoparticles

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ABSTRACT

A simple, sensitive and specific high-performance liquid chromatography (HPLC) assay for the quantification of camptothecin (CPT), a potent anticancer candidate, incorporated into solid lipid nanoparticles (SLN) in several rat organs (brain, heart, kidneys liver, lung, spleen) and serum was developed and validated. The sample pre-treatment involved organs homogenisation followed by CPT extraction. The samples were injected onto an analytical reversed-phase (RP) Mediterranea™ Sea18 column maintained at 30 °C. The chromatographic separation was achieved by gradient elution consisting of triethyamine buffer pH 5.5 and acetonitrile at a flow rate of 1.2 mL/min in 16 min of run time and retention time of 9.8 min (lactone). Fluorescence detection was used at the excitation and emission of 360 and 440 nm, respectively. The calibration curves in the different organs, serum and in PB3 were linear ($r^2 > 0.9999$) over CPT concentrations ranging from 1 to 200 ng/mL or 0.5 to 200 ng/mL ($n = 6$), respectively. The method was shown to be specific, accurate (between 94.4 ± 4.5% and 108.9 ± 0.6%) and precise at the intra-day and inter-day levels as reflected by the coefficient of variation (CV < 6.3%) at three different concentrations (10, 50 and 100 ng/mL) in all matrices. Stability studies showed that CPT was stable in all matrices after 24 h of incubation at room temperature (RT), after 24 h in the autosampler or after three freeze/thaw cycles. The mean recoveries of CPT in suspension, loaded into SLN and in a physical mixture with SLN at three concentrations of 10, 50 and 200 ng/mL were higher than 86.4%. The detection limit (DL) was ≤0.2 ng/mL and the quantification limit (QL) was ≤0.5 ng/mL. The method developed is reliable, precise and accurate and can be used in the determination of CPT amount in rat organ samples after i.v. administration of CPT in suspension, in physical mixture with SLN and incorporated in SLN.

1. Introduction

The natural alkaloid camptothecin (CPT) is a well-established topoisomerase I inhibitor against a broad spectrum of cancers [1,2]. Additionally, pre-clinical and clinical studies have shown the efficiency of CPT in malignant gliomas therapy [3–8]. Although, despite the strong anticancer activity, its *in vivo* use is highly limited due to poor aqueous solubility and instability of the lactone ring, conditioning its bioavailability, and rising toxic effects to normal tissues. The intact lactone ring of CPT is a crucial structure for their anticancer activity [9,10]. Unfortunately under physiological pH conditions (pH 7.4) the unstable CPT lactone ring readily opens to the carboxylate form, which is less cell-membrane permeable, possesses lower affinity for the target topoisomerase I, being therefore much less pharmacologically active (10-fold less) and simultaneously more toxic [2,9]. The CPT lactone-carboxylate equilibrium is reversible and pH-dependent (Fig. 1) with the lactone prevalence at acidic pH. At neutral and alkaline pH the carboxylate form is dominant.

To circumvent problems of rapid lactone hydrolysis and poor aqueous solubility, CPT is proposed to be incorporated into nanoparticles. One of the most promising nanoparticulate systems are solid lipid nanoparticles (SLN), submicron colloidal drug delivery systems with a mean size range usually between 100 and 400 nm [11], with a matrix composed of lipids being solid at room and body temperatures, dispersed in an aqueous surfactant solution, which combines the advantages of traditional colloidal carriers such as liposomes, polymeric nanoparticles and emulsions,

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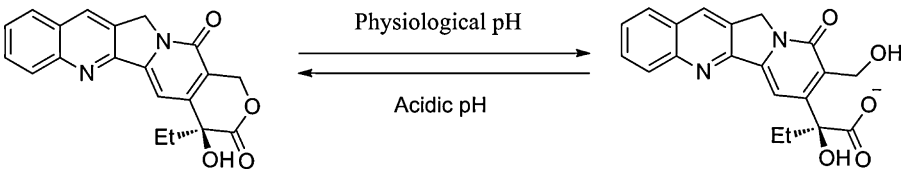

Fig. 1. CPT pentacyclic structure with a dynamic pH dependent equilibrium between the lactone (closed ring, left) and carboxylate (open ring, right) form of the drug.

but avoid or minimize the physical instability drawbacks associated with them [11,12]. The use of lipid and/or excipients of physiological or accepted status is an exceptional advantage once it decreases the risk of acute and chronic toxicity. Moreover, SLN are biodegradable and biocompatible, with low toxicity and easy to produce. These carriers have the suitability to incorporate both lipophilic and hydrophilic drugs within the lipid matrix in considerable amounts [11,12].

Moreover, the encapsulation of CPT into SLN appears to be advantageous for the stability of CPT. Yang et al. [13] showed that SLN greatly increased the stability of CPT towards hydrolysis and adequate retention of the active lactone, being SLN considered as a promising carrier for controlled release and targeted delivery of CPT. Since injectable formulations are difficult to achieve with the CPT lactone form due to its low water solubility, the poor *in vivo* stability of the drug turns SLN an attractive system for CPT delivery, because lipophilic nanoparticles are expected to chemically stabilise and solubilise CPT so that it is present in the active hydrophobic form and in therapeutically appropriate concentrations [13–15].

In order to produce the preclinical pharmacokinetic data of CPT-loaded SLN, it is required to develop an analytical method for CPT in biological matrices that could allow the accurate and precise determination of the drug at therapeutic concentrations, for a wide range and number of biological samples. Some methods, based on HPLC with fluorescent detection, have already been described for the detection and quantification of CPT [16–20] and CPT analogues [21–27] in biological matrices, but those methods demonstrated relatively high limits of quantification, which may limit their application. Despite that, to the best of our knowledge, no study reported was so complete and analyse as many different biological matrices as are analysed in the present paper. Therefore, the main objective of the present work was to develop and validate a simple and sensitive HPLC method with a sufficiently low quantification limit to support pharmacokinetic and bioequivalence studies of CPT-loaded SLN.

The method described in this paper is a simple, accurate HPLC method to quantify CPT in several organs with fluorescence detection, fully validated according to ICH guidelines [28]. Furthermore, it gives information about the stability of CPT both in the matrices and during sample processing (24 h at room temperature (RT), 24 h in the autosampler of the HPLC device, and three freeze–thaw cycles), which is a noticeable advantage for assessing a large number of samples for pharmacokinetic and bioequivalence studies *in vivo*.

2. Experimental

2.1. Chemical and reagents

The wax cetyl palmitate (CP) was a gift from Gattefossé SA, (France). The surfactant polysorbate 80 (P80) was provided by Merck KgaA (Germany). (S)-(+)-Camptothecin (CPT) (with a purity of ~95%) was provided by Sigma–Aldrich (Portugal). All organic solvents were of HPLC grade. Acetic acid was obtained from Pronalab (José M. Vaz Pereira Lda, Portugal). Acetonitrile was procured from Panreac Químicas S.A.U. (Spain). Dimethyl sulfoxide (DMSO) and di-sodium hydrogen phosphate anhydrous were obtained from Merck KgaA (Germany). Ortho-phosphoric acid 85% (w/w) was provided by Panreac Químicas S.A.U. (Spain). Triethylamine (TEA) was obtained from VWR International S.A.S. (France). Purified water was of MilliQ®-quality prepared in our laboratory.

2.2. Solid lipid nanoparticles production

Formulations containing the lipid CP and the surfactant P80 were prepared at concentrations of 5% (w/w) of lipid and 2% (w/w) of surfactant by the high shear homogenisation and ultrasonication (US) techniques. Briefly, the lipid and surfactant mixture was melted at approximately 5–10 °C above the melting point of the lipid (CP melting point around 57 °C). MilliQ water was heated to approximately the same temperature and transferred to the surfactant lipid mixture. A pre-emulsion consisting of lipid and surfactant in water was prepared with an ultra-turrax T25 (IKA-Labortechnik, Germany) at 8000 rpm during 30 s, and an US probe at 70% amplitude for 2.5 min (VibraCell model VCX 130 equipped with a 6 mm probe, Sonics & Materials, Inc., Newtown, CT, USA). CPT-loaded SLN was prepared by adding the drug (0.1% w/w) to the molten lipid prior to particle preparation.

2.3. Physicochemical characterisation of solid lipid nanoparticles

Particle size and distribution (polydispersity index, PI) was determined by dynamic light scattering (DLS), using a Zetasizer Nano ZS laser scattering device (Malvern Instruments Ltd., Malvern, UK). The samples were diluted with water with a conductivity adjusted to 50 μS/cm, by dropwise addition of 0.9% (w/v) NaCl solution, and analysed. The Zetasizer Nano Series V6.20 software was used to correlate the intensity of scattered light (at a backscattering angle of 173°) with the hydrodynamic radius of the spherical particle. Several dilutions of the sample were tested at 25 °C to obtain dispersed and isolated nanoparticles in the solvent. For each sample, the mean of at least three determinations (±standard deviation) was calculated applying multimodal analysis.

The electrophoretic mobility (zeta potential (ZP)) of the nanoparticles and ultimately their surface charge was measured by combining laser Doppler velocimetry and phase analysis light scattering (PALS) using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). The samples, diluted in water with conductivity adjusted to 50 μS/cm by dropwise addition of 0.9% (w/v) NaCl solution, were placed in polystyrene cuvettes with platinum electrodes and then an electric field was applied across the dispersion of the nanoparticles. Surface charged particles within the dispersion migrated towards the electrode of opposite charge and the velocity of particles migration was converted in ZP values by using the Smoluchowski's equation. The ZP results reported are the mean of at least three determinations (±standard deviation).

CPT association efficiency (AE) was determined by HPLC upon separation of CPT-loaded SLN from free CPT by centrifugation (Centrifuge 5804 with rotor A-4-44, Eppendorf, Spain) using centrifugal filter units (Amicon Ultra, ultracel 50 K, Milipore, Carrigtwohill, Co. Cork, Ireland). Prior to centrifugation (1500 × *g*, 20 min), SLN were diluted 100 times in MilliQ water. Following centrifugation, the supernatant was collected, further diluted 20 times in PB3 and

analysed by HPLC as described below. CPT concentration in SLN was detected indirectly by HPLC according to Eq. (1):

$$AE = \frac{\text{Total amount of CPT} = \text{Free CPT in supernatant}}{\text{Total amount of CPT}} \times 100 \quad (1)$$

2.4. Analytical procedure

2.4.1. Instruments and analysis conditions

A Shimadzu UFLC Prominence System (USA) equipped with two Pumps LC-20AD, an autosampler SIL-20AC, a column oven CTO-20AC, a degasser DGU-20A5, a System Controller CBM-20A and a LC Solution, Version 1.24 SP1 (Shimadzu) was used in all chromatographic analysis. The Shimadzu RF-10Axʟ fluorescence detector (FD) coupled to the LC System was used for peak area detection. The chromatographic analysis was performed at 30 °C on an analytical reversed-phase (RP) Mediterranea™ Sea18 column (150 mm × 4.0 mm, 5 μm, Teknokroma, Spain) protected with a precolumn Ultraguard™ (Guard column Sea18, 10 mm × 3.2 mm, Teknokroma, Spain).

The optimised method used a binary gradient mobile phase with 1% (v/v) triethylamine buffer at pH 5.5 (pH adjusted with acetic acid) as mobile phase A and acetonitrile as mobile phase B. A flow rate of 1.2 mL/min was used with a 10 μL injection volume. The program started with a gradient of 75%A and 25%B and after 1 min the gradient changed continuously until the minute seven were it reaches the gradient of 40%A and 60%B which was maintained until minute nine. Afterwards, the gradient was changed again to 75%A and 25%B and remained constant until minute sixteen.

The eluted peaks were monitored at excitation and emission wavelengths of 360 and 440 nm, respectively.

2.4.2. Preparation of stock and standard solutions

Stock solutions of CPT were prepared daily in DMSO at concentration of 1 mg/mL and further diluted in 9 mM phosphate buffer, pH 10.5 (PB10.5) and pH3 (PB3) to a concentration of 10 μg/mL. Standard solutions were prepared by dilution in PB3 (lactone CPT-standards) or PB10.5 (carboxylate CPT-standard) to a final CPT concentration between 0.5 and 200 ng/mL.

The QC samples at three different levels (low, middle and high) were prepared as a single batch at each concentration (10, 50 and 100 ng/mL) in PB3.

2.4.3. Preparation of biological matrices sample

Firstly, a mixture of PB 3 and 0.15 M phosphoric acid (1:1, v/v) was added to each organ at the same amount (1:1, w/w), and homogenised using an ultra-turrax. Secondly, 200 μL of phosphoric acid (0.15 M) and 800 μL of acetonitrile were added to 0.2 g of the homogenate. This mixture was vigorously vortexed for 1 min, followed by centrifugation at 3000 rpm for 15 min to isolate the supernatant. The filtered supernatant (150 μL) was mixed with 150 μL of PB3 standard solution to a final CPT concentration between 1 and 200 ng/mL. The resultant sample (300 μL) was analysed for CPT content by HPLC.

The QC samples at three different levels (low, middle and high) were prepared as a single batch at each concentration (10, 50 and 100 ng/mL) by adding 150 μL of the appropriate standard solution in PB3 in 150 μL of the appropriated biological matrix.

2.5. Method validation

The chromatographic method was validated for specificity, linearity, accuracy, precision, range and robustness according to ICH guideline [28].

2.5.1. Selectivity

Selectivity of the method was determined by analysing six different samples of blank PB, serum brain, blood, heart, kidneys, liver, lungs or spleen obtained from healthy rats.

2.5.2. Linearity and range

To evaluate linearity, calibration curves were prepared and analysed in triplicate on three consecutive days. Linearity was determined by calculation of a regression linear line from the peak area vs. concentration plot for eight standard solutions in PB3 (0.5, 1, 2.5, 10, 25, 50, 100 and 200 ng/mL) or six standard solutions spiked in biological matrices (1, 2.5, 10, 50, 100 and 200 ng/mL) using linear least squares methodology, and by analysis of the respective response factors (i.e., peak area divided by concentration of each standard sample).

2.5.3. Accuracy and precision

The accuracy and precision were also determined by replicate analyses ($n = 6$) of QC samples at three concentration levels (10, 50 and 100 ng/mL) followed by their comparison with the calibration curves prepared on the same day and on different days to assess intra-day and inter-day variation, respectively. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100 (%) and the precision by the coefficient of variation (CV = (standard deviation/mean) × 100%). The concentration of each sample was determined using the calibration curve and analysed on the same day.

2.5.4. Detection limit and quantification limit

Detection limit (DL) and quantification limit (QL), for each matrix, were calculated according to Eqs. (2) and (3), respectively:

$$DL = \frac{3.3\sigma}{S} \quad (2)$$

$$QL = \frac{10\sigma}{S} \quad (3)$$

where σ = the standard deviation of the response, S = the slope of the calibration curve.

2.5.5. Robustness

2.5.5.1. Stability. The short-term stability was assessed by maintaining the QC samples at RT for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at RT for 2 h and refreezing for 24 h. Autosampler stability of CPT was tested by analysis of QC samples, which were stored in the autosampler tray of the HPLC instrument for 24 h. For each concentration and each storage condition, six replicates were analysed in one analytical batch. The concentration of CPT after each storage period was compared to the initial concentration determined for the samples that were freshly prepared and processed immediately.

2.5.5.2. Extraction recovery. The absolute extraction recoveries of CPT from biological matrices at three QC levels were determined by assaying the samples as described in Section 2.4.3 and comparing the peak areas of the CPT with those obtained from direct injection of the analyte spiked in the same amount in the supernatant of the processed blank matrices.

2.6. Application of the method to pharmacokinetic studies

Healthy male Wistar rats (250–300 g) used for pharmacokinetic studies were obtained from Charles River (Spain). Animal handling and procedures used were according to standard operating procedure approved in the ETS 123-European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and approved by Direcção Geral de Veterinária

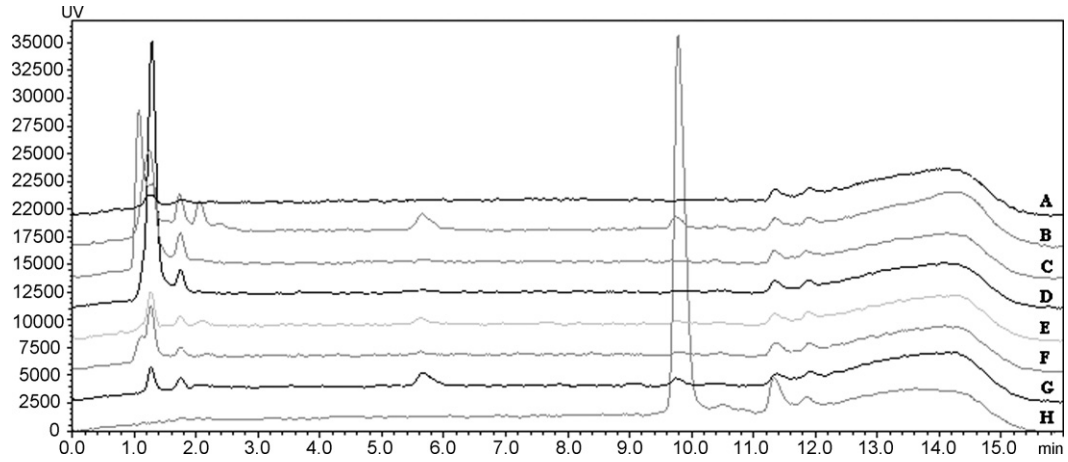

Fig. 2. Chromatograms of blank brain (A), heart (B), kidneys (C), liver (D), lungs (E), spleen (F), serum (G) and CPT in PB3 (10 ng/mL) (H).

(DGV, Portugal). The different formulations (CPT in suspension, CPT-loaded SLN and physical mixture of CPT and unloaded SLN) were administered intravenous via the lateral tail vein (0.5 mg/kg). The animals were sacrificed 1 h after CPT injection with lethal dose of anaesthesia. The total blood was removed by exsanguination and the organs were collected and preserved in ice during all the procedure.

The concentration of CPT in the biological matrices (serum, brain, heart, kidneys, liver, lungs and spleen) was determined by processing the samples as described and further analysing the CPT content by the developed and validated HPLC method. Groups of 6 healthy male Wistar rats were chosen for the pharmacokinetic study for each formulation. Three formulations (CPT in suspension, CPT-loaded SLN and physical mixture of CPT and unloaded SLN) were prepared immediately before the experiment and injected in the lateral tail vein at a final concentration of 0.5 mg/kg of rat (~125–150 μL). CPT-loaded SLN were prepared as described above, the physical mixture of CPT and empty SLN were prepared by adding the CPT to unloaded SLN (1 mg/g) followed by 20 min of sonication in a sonicating water bath (Bandelin SONOREX RK100H, Bandelin Electronic GmbH & Co. KG, Germany). CPT suspension was prepared according a procedure developed by Fox et al. [29] to assure the redispersibility and distribution of CPT. Previous to the CPT suspension, 3.6 g of P80, 32.4 g of purified water and 4 g of NaCl solution (0.9%, w/v) were mixed and CPT was suspended in the previous solution (1 mg/g) and sonicated for 20 min in a sonicating water bath.

2.7. Statistical analysis

For the SLN physicochemical characterisation results are shown as the mean ± standard deviation (SD) of at least 3 different batches of the same formulation. The *t*-test and the one-way analysis of variance (ANOVA) were performed to compare two or multiple groups, respectively. If the group by each time interaction was significantly different ($P < 0.05$), differences between groups were compared within a post hoc test (Tukey HSD). To describe statistical differences between results obtained with CPT in suspension (control) and CPT incorporated into SLN or in physical mixture with SLN the Dunnett test was used. All statistical analyses were performed with the software PASW Statistic 18 (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Physicochemical characterisation of solid lipid nanoparticles

Unloaded SLN and CPT-loaded SLN were produced and characterised in terms of mean diameter, PI and ZP on the day of production. Unloaded and CPT-loaded SLN had similar mean sizes of 148.70 ± 7.92 nm and 159.71 ± 3.0 nm, respectively, and PI values of 0.208 ± 0.040 and 0.156 ± 0.013, respectively. The ZP values, slightly negative for all the formulations, were −18.77 ± 2.4 mV (unloaded) and −24.02 ± 2.86 mV (CPT-loaded SLN). CPT was incorporated with a higher AE of 96.5%.

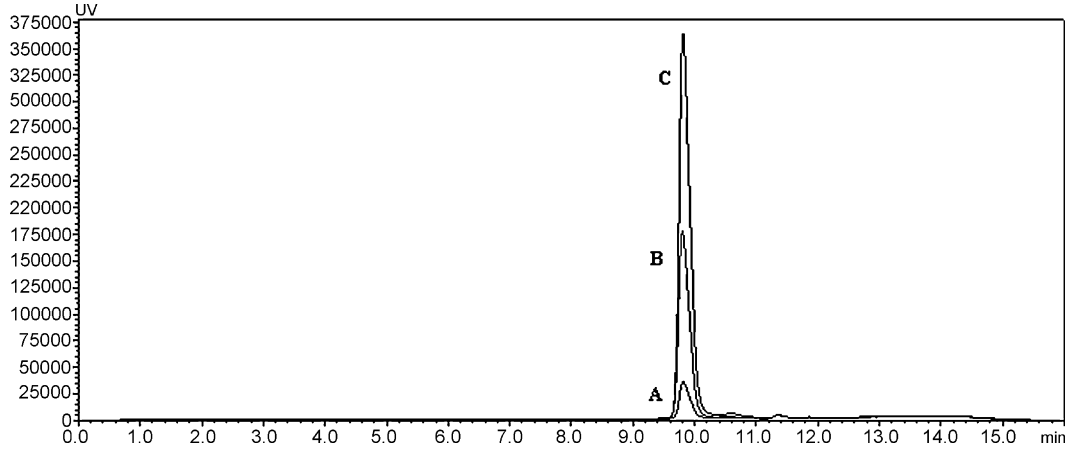

Fig. 3. Chromatogram of CPT in PB3 (10 (A), 50 (B) and 100 (C) ng/mL).

According to the literature [30], the mean diameter of SLN seems to be suitable for reaching cancer cells, to be taken up and to release drug inside cells. In relation to the charge surface, unloaded and CPT-loaded SLN had slightly negative charge, and these values of ZP are good indications also for brain tumour targeting [30,31]. Another advantage of using negatively charged SLN is that they are expected to be less toxic and more stable compared to positively charged nanoparticles [32]. Based on these facts, we propose that the currently developed formulations with a slightly negative charge may not exert surface associated cytotoxicity and increase the drug delivery of incorporated drugs to the brain.

3.2. Chromatography

The validation of the analytical HPLC method was carried out calculating selectivity, linearity, intra-day and inter-day precision and accuracy, extraction recovery and stability. These are mandatory parameters proposed by the Authorities and desired by Pharmaceutical Industry in order to obtain better and more safety medicines.

Selectivity is the capability of an analytical method to distinguish and quantify the analyte in the presence of other inherent components in the samples [33]. For selectivity, analyses of blank samples (6 replicates) of all the matrices were performed.

Fig. 2 represents chromatograms of blank matrices samples and CPT in PB3 (10 ng/mL). It was established that the detection of the analyte was not interfered, in chromatographic separation, by endogenous compounds present in the serum or organs samples since no peaks at the CPT retention time were detected (Fig. 2), being any potential interfering compound washed from the column together with the solvent.

To evaluate linearity, calibration curves were constructed by plotting the peak area of each analyte against the concentration of the analyte. Data were fitted to least squares linear regression. In a binary gradient mobile phase with 1% TEA in water and acetonitrile, the assay demonstrated good linearity in the tested range for CPT, in all the matrices ($R^2 > 0.999$). Under the present chromatographic conditions, the run time for each sample was 16 min. The retention times were 3.0 and 9.8 min for CPT, carboxylate and lactone form, respectively, as demonstrated by the analyses of CPT solutions at different pH (data not shown). No CPT carboxylate peaks were detected in the organs due to the low pH (pH∼3) of the samples after pre-treatment that transforms any CPT carboxylate form in the lactone form (acid-lactone inter-conversion) (Fig. 3). Thus, in this work, no calibration curve was presented for the carboxylate CPT form.

Regression analysis of calibration curves for PB3, serum, brain, heart kidneys, liver, lung, and spleen, over the specified concentration rate, are depicted in Table 1. Calibration curve ($n = 6$) for CPT in PB3 was linear and reproducible over the concentrations examined (0.5–200 ng/mL). Regression coefficient (R^2) was greater than 0.999. The suitable range for CPT analyses from 0.5 to 200 ng/mL was also demonstrated by the response factors versus concentration (Fig. 4), since the linear curve slope of approximately zero (slope = 0.1132) indicate that a linear response is obtained over the specified concentration range. The calculated DL of CPT was 0.2 ng/mL and the QL was 0.5 ng/mL. The QL value found revealed a precision expressed by % CV equal to 7.3% and accuracy of 99.9%. According to FDA guidelines the QL found had an acceptable precision (i.e., CV < 20%) and accuracy (i.e., accuracy 100 ± 20%) [33].

Calibration curves for CPT in biological matrices (serum, brain, heart kidneys, liver, lung, and spleen) were also linear for the range tested (1–200 ng/mL) with a regression coefficient (r^2) higher than 0.999 (Table 1). The DL and QL limits of the biological matrices were ≤0.18 and ≤0.54, respectively (Table 1). Despite the low calculated QL for some biological matrices, these values will probably be associated with a low precision and accuracy. For the purpose of this work we only considerate as measurable CPT concentrations values, in biological matrices, higher than 1 ng/mL, since they have acceptable precision (CV between 4.7% and 7.5%) and accuracy (accuracy between 95.3% and 104.3%) values.

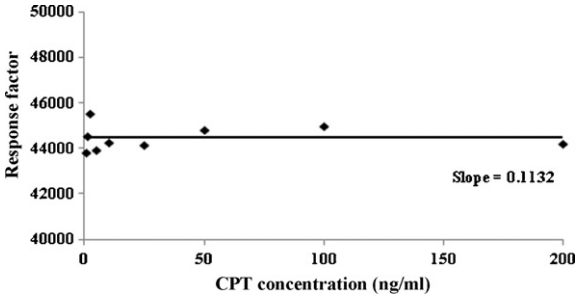

Fig. 4. Linearity studies for the proposed HPLC method. Response factors versus CPT standard solutions concentration.

Accuracy and precision (inter- and intra-day) were also estimated. The accuracy of an analytical method depicts the closeness of the mean results achieved by the method to the nominal concentration of the analyte [33]. The precision of an analytical method represents the closeness of individual measures of an analyte when the procedure is used repetitively to multiple aliquots of a single homogenous volume of matrix [33].

The intra-day precision and accuracy were calculated by analysing the QC of all matrices at concentrations of 10, 50 and 100 ng/mL. The intra-day precision was ≤2.7% and accuracy ranged between 94.4 and 102.2% (Table 2).

The inter-day precision and accuracy were calculated by analysing the QC of all matrices at concentrations of 10, 50 and 100 ng/mL. The intra-day precision was ≤ 6.3 and accuracy ranged between 99.4 and 108.9% (Table 2).

Both intra- and inter-day precision and accuracy found have an acceptable precision and accuracy, since according to FDA guidelines the precision and accuracy found for each concentration level should not exceed 15% of the CV and accuracy range should be between 85 and 115% [33].

In order to evaluate the robustness of the developed method, it was performed stability and recovery assays. The stability of CPT at laboratory temperature was assessed analysing fresh samples and analysing the same samples after 24 h at RT. CPT shown stability in all matrices after this period as can be seen from Table 3.

The autosampler stability was assessed analysing fresh samples and analysing the same samples after 24 h after the first injection. CPT was stable in the autosampler for at least 24 h (Table 3.)

The freeze–thaw stability of CPT was over three freeze–thaw cycles, as can be seen in Table 3, and all the matrices show stability after this period.

The recoveries of CPT from biological matrices were estimated comparing the peak areas obtained with those obtained from the injections of biological samples spiked with known concentrations of the analyte. For CPT recoveries values ranged from 86.4 and 99.9% and CV values between 0.3 and 9.5% were obtained (Table 4). These values are in good agreement with a high degree of extraction of drugs from organs, which could be a limiting step for the correct pharmacokinetics profile. And for this validation, the use of an internal standard was considered since the linearity of the calibration curves in all the matrices was highly acceptable ($R^2 > 0.999$) and the intra-day and inter-day precision and recoveries of the assay in measuring control samples without internal standards was adequate. This is also in agreement with similar HPLC methods [24].

The comparison between the developed method and similar methods for CPT quantification in biological matrices is depicted

Table 1
Regression analysis of calibration curves, detection limit (DL) and quantification limit (QL) for CPT in PB3, serum, brain, heart kidneys, liver, lungs, spleen, over the specified concentration rate.

Matrix	Range (ng/mL)	Slope	Intercept	Correlation coefficient (r)	DL (ng/mL)	QL (ng/mL)
PB3	0.5–200	44469	1008	0.9999	0.17	0.50
Serum	1–200	46767	−44574	0.9999	0.12	0.37
Brain	1–200	44276	−25805	0.9999	0.06	0.17
Heart	1–200	47652	75468	0.9999	0.18	0.54
Kidneys	1–200	48389	−47525	0.9999	0.04	0.12
Liver	1–200	51417	−87933	0.9999	0.03	0.10
Lungs	1–200	51319	−41806	0.9999	0.05	0.17
Spleen	1–200	44333	−57379	0.9999	0.11	0.32

Table 2
Accuracy and precision for the analysis of CPT in PB3, serum, brain, heart kidneys, liver, lungs, spleen ($n = 6$).

Matrix	Nominal concentration[a] (ng/mL)	Intra-day			Inter-day		
		Measured concentration			Measured concentration		
		Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
PB3	10	10.1	100.9	1.4	10.1	100.7	1.3
	50	51.2	102.5	0.1	51.0	102.1	0.1
	100	101.7	101.7	0.6	101.3	101.3	0.6
Serum	10	9.4	94.4	4.8	10.7	107.4	1.0
	100	101.6	101.6	2.0	107.1	107.1	1.1
Brain	10	10.7	106.6	1.9	10.1	100.7	1.8
	100	99.2	99.2	1.4	99.4	99.4	1.4
Heart	10	9.8	98.2	1.2	10.2	102.5	1.1
	100	100.8	100.8	0.8	100.4	100.4	1.7
Kidneys	10	10.0	100.0	1.5	10.0	99.8	0.5
	100	100.4	100.4	2.2	100.3	100.3	1.5
Liver	10	10.3	102.5	2.2	10.2	102.0	4.2
	100	102.2	102.2	4.1	101.8	101.8	6.3
Lungs	10	9.7	97.3	2.4	10.8	107.9	2.6
	100	100.8	100.8	1.8	100.9	100.9	5.4
Spleen	10	9.7	96.7	1.2	10.8	108.9	1.0
	100	101.1	101.1	2.7	100.1	100.1	4.3

[a] Selected concentrations represent the low and high quality control concentrations. Intra-day and inter-day accuracy and precision was determined with replicates ($n = 6$) for each concentration. CV (coefficient of variation) = (SD/mean) × 100.

in Table 5. The proposed method is suitable for measuring CPT in 7 different biological matrices, when generally the other methods are suitable only for quantify CPT in one or two biological matrices. Additionally, the present method revealed generally lower DL and QL values, higher recoveries and appropriate stability of the samples, which are, undoubtedly, clear advantages for perform pharmacokinetic studies.

3.3. Application of the method to pharmacokinetic studies

The proposed method was used to assess the rat body distribution of CPT after i.v. administration. Results from pharmacokinetic studies are depicted in Table 6. A complete pharmacokinetic study of the CPT using different formulations of CPT-loaded SLN during 24 h was also performed after the method validation, giving a more

Table 3
Stability of CPT at different experimental conditions in PB pH 3, serum, brain, kidneys, liver, heart, kidneys, liver, lungs and spleen ($n = 6$).

Experimental condition		24 h at RT		24 h in the autosampler		3 freeze–thaw cycles	
Matrix	Nominal concentration[a] (ng/mL)	Measured concentration					
		Mean (ng/mL)	CV (%)	Mean (ng/mL)	CV (%)	Mean (ng/mL)	CV (%)
PB3	10	11.1	2.7	9.5	2.3	11.4	4.4
	100	104.6	8.7	98.7	0.2	101.3	1.1
Serum	10	10.3	2.4	9.1	1.5	10.9	0.1
	100	103.6	3.6	96.4	0.8	105.6	0.5
Brain	10	10.2	0.5	10.5	1.8	10.3	1.6
	100	103.3	4.3	96.4	0.2	101.5	1.5
Heart	10	11.1	2.8	9.3	1.6	10.5	1.2
	100	105.0	2.8	96.7	0.3	97.9	0.7
Kidneys	10	11.3	2.1	9.2	1.7	10.0	5.2
	100	102.5	3.2	93.8	2.8	97.9	1.3
Liver	10	9.9	0.6	9.3	0.7	11.2	9.3
	100	90.1	1.0	89.4	0.6	93.3	5.6
Lungs	10	10.9	0.6	9.4	0.7	9.7	1.6
	100	100.4	4.0	96.4	0.2	91.2	0.5
Spleen	10	10.9	2.3	10.3	1.5	10.1	0.8
	100	97.6	0.1	105.1	1.1	99.4	0.1

[a] Selected concentrations represent the low and high quality control concentrations. Stability of CPT at different experimental conditions was determined with replicates ($n = 6$) for each concentration. CV (coefficient of variation) = (SD/mean) × 100.

Table 4
Extraction efficiency of CPT from rat organs at different concentration levels ($n = 6$).

	CPT solution			CPT-loaded SLN		SLN + CPT	
	T.C.	E.E. (%)	CV (%)	E.E. (%)	CV (%)	E.E. (%)	CV (%)
Serum	10	97.9	2.3	86.8	4.9	91.6	9.5
	50	97.3	3.7	86.7	0.3	86.7	0.3
	100	94.2	1.1	90.3	1.8	99.1	0.8
Brain	10	94.8	4.2	94.5	1.3	96.0	6.1
	50	98.3	4.5	93.2	0.5	99.9	7.6
	100	94.7	3.3	96.3	0.2	90.9	3.2
Heart	10	94.2	3.9	97.1	2.6	98.0	1.4
	50	95.2	0.5	98.6	0.2	90.3	5.7
	100	96.7	4.9	86.4	0.5	93.9	1.1
Liver	10	87.0	1.6	93.6	0.8	89.5	2.9
	50	95.3	3.1	96.5	3.0	94.8	3.9
	100	95.2	5.6	92.5	2.2	95.4	4.0
Lungs	10	88.7	1.8	89.7	0.9	96.1	0.2
	50	89.1	1.9	92.2	2.0	99.7	1.8
	100	95.4	1.1	96.5	1.9	95.3	0.6
Kidneys	10	95.6	1.4	87.2	1.3	95.5	1.1
	50	98.3	1.1	90.2	1.6	96.3	4.6
	100	93.8	4.4	97.7	1.4	96.7	2.5
Spleen	10	97.7	4.9	90.8	2.7	91.6	2.4
	50	97.1	1.0	97.0	2.1	94.8	4.1
	100	96.6	1.7	91.3	0.3	91.4	0.4

Table 5
Parameters comparison between the proposed method and other methods for the detection of camptothecin.

Biological matrices	Time of run (min)	Equilibrium time (min)	DL (ng/mL)	QL (ng/mL)	Stability	Recovery (%)	Ref.
Serum			0.12	0.37		>86	
Brain			0.06	0.17		>90	
Heart			0.18	0.54	24 h autosampler	>90	
Kidneys	16	–	0.04	0.12	24 h RT	>87	This work
Liver			0.03	0.10	3 freeze/thaw cycles	>88	
Lungs			0.05	0.17		>87	
Spleen			0.11	0.32		>90	
Plasma	>12	n.a.	0.5	1	n.a.	>62	[20]
Urine				10		>55	
Plasma	18	3–5	n.a.	5	Month storage −80 °C 4 h RT 3 freeze/thaw cycles	>76	[16]
Plasma	7.5	n.a.	n.a.	5.74 nM	24 h at −70 °C, 4 °C or 37 °C1 month stored at −70 °C	>80	[17]
Plasma	>16	n.a	0.2	n.a	1 month at −30 °C	>78	[18]

precise outlook of the potentiality of SLN carriers of drugs for brain target (unpublished data). CPT distribution 1 h after CPT administration revealed that the concentration in some organs clearly increased when CPT was incorporated into SLN, mainly in the brain, where the CPT accumulation was greatly increased. CPT accumulation was increased in brain (6.3 times, $p < 0.05$), kidneys (2.5 times, $p < 0.05$), liver (1.4 times), lungs (2.4 times, $p < 0.05$), spleen (2.0 times, $p < 0.05$) and serum (2.8, $p < 0.05$). This increase is probably due to a higher delivery of the drug (e.g. brain) and/or to a lower elimination rate from the rat body. In agreement with the increment of CPT levels it was also verified that the total CPT concentration measured in all the organs were 2.9, 5.9 and 5.3 μg/g for CPT in suspension, CPT-loaded SLN and physical mixture of CPT in SLN, respectively. On the other hand, CPT concentration decreased in the heart (0.7 times, $p < 0.05$) in comparison with CPT in suspension, which may be a consequence of the low affinity of the SLN containing CPT to the heart and to the deposition of SLN containing the drug in other organs, thus less CPT in heart. The lower affinity of SLN to the heart may also be beneficial to reduce cardiotoxicity of some drugs, such as doxorubicin [34,35].

Furthermore, CPT accumulation was higher, when incorporated in SLN than when in physical mixture with SLN, in brain (4.0 times, $p < 0.05$), in the lungs (2.1 times, $p < 0.05$) and in the serum (1.6 times, $p < 0.05$). This increase demonstrate the ability of SLN to modulate and target the rational pharmacokinetics of CPT. On the other hand, CPT concentration decreased in the heart (0.5 times,

Table 6
Concentration of CPT (ng/g organ) in rat 1 h after i.v. administration of CPT suspension, CPT-loaded SLN and physical mixture of CPT and unloaded SLN ($n = 6$).

Organ	CPT suspension		CPT-loaded SLN		CPT + SLN	
	Mean	SD	Mean	SD	Mean	SD
Serum	32.4	7.7	89.5*	18.8	54.4	10.7
Brain	38.8	7.5	242.6*	69	61.3	8.8
Heart	75.2	6.5	50.9	11.9	102.6*	7.2
Kidneys	44.8	9.9	114.0	23.3	170.6*	39.4
Liver	873.8	265.7	1229.2	309.7	1774.5*	444.5
Lungs	1378.8	463.7	3248*	817.9	1575.9	485.8
Spleen	468.2	126.1	949.4	226.1	1529.9*	309.6

* Significantly different ($P < 0.05$) from the results obtained with CPT in suspension formulation.

$p < 0.05$), in the kidneys (0.7 times, $p < 0.05$), in the liver (0.7 times, $p < 0.05$), in the spleen (0.6, $p < 0.05$) in comparison with CPT in physical mixture with SLN which may be a consequence of the targeting of SLN containing the drug incorporated to the brain and lungs with the decreasing accumulation in other organs.

CPT-loaded SLN seems to be more suitable to target CPT to brain and lungs than CPT in suspension or CPT in physical mixture with SLN. SLN containing the drug revealed a higher systemic concentration after 1 h than the other two formulations, meaning that the drug is able to circulate at higher concentration and during longer time through the body. It is well know that the tumour vasculature is immature, allowing nanoparticles leaking from the blood to accumulate in the interstitial space in tumour tissues due to the "enhanced permeability and retention (EPR) effect" [36]. Consequently, the increase of circulation of CPT incorporated into SLN is an enormous advantage since increased systemic exposure results generally in increased tumour exposure to the drug, and can result in high accumulation in the tumour.

4. Conclusion

A simple, sensitive, accurate and precise HPLC method was developed and fully validated according to the ICH guidelines for analytical procedures validation. This method was convenient for the quantification of CPT in rat organ samples and was successfully applied to the determination of biodistribution of CPT in rat. This method, based in a chromatographic separation and fluorescent detection, could be very useful for clinical pharmacokinetic studies in other animals and humans.

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