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Simple liquid chromatography method for the quantification of irinotecan and SN38 in sheep plasma: Application to in vivo pharmacokinetics after pulmonary artery chemoembolization using drug eluting beads

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ABSTRACT

A rapid and simple liquid chromatography–fluorescence detection (LC–FD) method was developed and validated for the simultaneous quantification of irinotecan (CPT11) and SN38 in sheep plasma. Camptothecin (CPT) was used as the internal standard. A single step protein precipitation with acetonitrile was used for sample preparation. The separation was achieved using a 5 μm C18 column (250 mm × 4.5 mm, 5 μm) with a mobile phase composed of 36 mM sodium dihydrogen phosphate dehydrate and 4 mM sodium 1 heptane sulfonate–acetonitrile (72:28), the pH of the mobile phase was adjusted to 3. The flow rate was 1.45 mL/min and the fluorescence detection was operated at 355/515 nm (excitation/emission wavelengths). The run time was 13 min. The method was validated with respect to selectivity, extraction recovery, linearity, intra- and inter-day precision and accuracy, limit of quantification and stability. The method has a limit of quantification of 5 ng/mL for both CPT11 and SN38. The assay was linear over concentrations ranging from 5 to 5000 ng/mL and to 240 ng/mL for CPT11 and SN38, respectively. This method was used successfully to perform plasma pharmacokinetic studies of CPT11 after pulmonary artery embolization (PACE) in a sheep model. It was also validated for CPT11 and SN38 analysis in sheep lymph and human plasma.

1. Introduction

Irinotecan (CPT11) is a potent inhibitor of topoisomerase I. It is one of the key drugs of anticancer chemotherapy, especially for the treatment of colorectal and lung tumors [1,2]. CPT11 is a prodrug converted in vivo in humans by carboxylesterases to an active metabolite SN38 (Fig. 1). Both CPT11 and SN38 are prone to interconversion between lactone form and carboxylate form. The closed lactone configuration of SN38 is the pharmacological active form [3].

Drug eluting beads (DEB) are a new generation of embolization beads which behave as a drug delivery system [4]. They are used in chemoembolization which is a promising therapeutic technique for treating cancers [5–8]. Chemoembolization is an alternative strategy to increase the local drug concentration and reduce systemic toxicity. This non-surgical technique consists of delivering directly to the tumor high concentrations of chemotherapeutic agents which are combined with or loaded in embolization beads. The purpose of embolization beads is to achieve nutrient and oxygen starvation of the tumor and to minimize chemotherapy wash-out with prolonged contact with tumor tissue. DEB can be loaded with CPT11 (DEB–CPT11) and administered intra-arterially [4,8]. This drug device combination may offer the possibility to precisely control the release and the dose of drug into the tumor bed.

DEB–CPT11 have been evaluated successfully in preclinical and clinical studies for hepatic embolization [8–11]. No preclinical or clinical study with DEB–CPT11 was published for lung embolization. For a preclinical study evaluating DEB–CPT11 for pulmonary artery chemoembolization (PACE), we have chosen sheep, a classical model for embolization [12–14]. Sheep is a suitable model for lung embolization because of its similarities with human lung physiology and architecture. Furthermore, the same medical devices and

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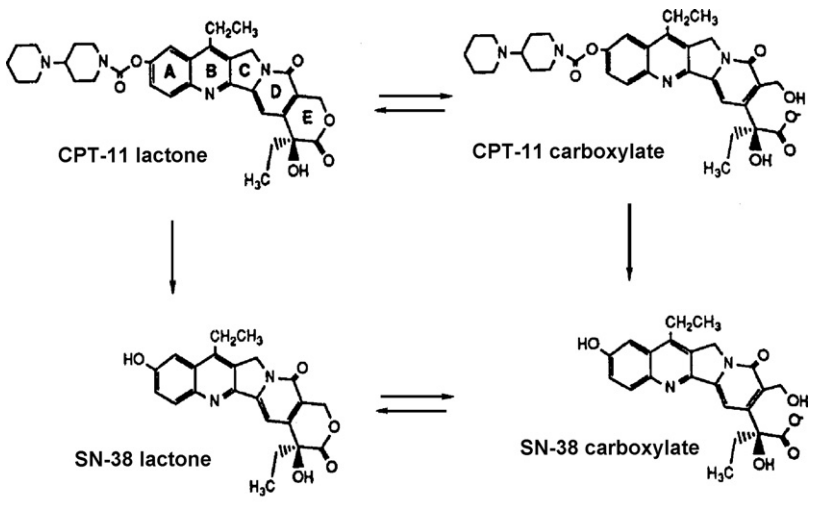

Fig. 1. Chemical structures of irinotecan and SN38 (lactone and carboxylate forms).

DEB size are used for embolization procedures. Hence, with sheep, we are in nearest human situations for PACE.

Our preclinical study purpose is first to evaluate in vivo the safety and efficacy of CPT11 release from DEB in a sheep lung embolization model. To the best of our knowledge, no available method is published for the determination of CPT11 and SN38 in sheep plasma.

Liquid chromatography (LC) methods have already been described for the determination of CPT11 and SN38 in plasma. Most of the published methods include long extraction procedures with solid-phase extraction or multi-step liquid–liquid extraction including evaporation step [15–18] or require specific and expensive equipment as mass spectrometer or fluorescence detector with automatically switch wavelengths [19–21]. The aim of this study was to develop and validate a sensitive, rapid and simple LC method for CPT11 and SN38 quantification using standard laboratory equipment, in order to study CPT11 and SN38 pharmacokinetics in sheep plasma after PACE with DEB. The assay was applied to a preclinical study. It was also successfully applied to sheep lymph and human plasma analysis and will be used in further studies.

2. Experimental

2.1. Chemicals and reagents

Irinotecan solution (Campto® 20 mg/mL, Pfizer, France) was used to prepare standards. Camptothecin (95% purity) was purchased from Sigma–Aldrich (St Quentin Fallavier, France) and used as the internal standard (IS). SN38 was supplied by Pfizer (USA). HPLC grade acetonitrile was obtained from Sigma–Aldrich (St Quentin Fallavier, France). Sodium 1 heptane sulfonate and sodium dihydrogen phosphate dehydrate were of analysis grade purchased from Sigma–Aldrich (St Quentin Fallavier, France). HCl 37% (hydrochloric acid) Normapur® and H_3PO_4 85% (orthophosphoric acid) Normapur® were obtained from VWR Prolabo (Fontenay sous bois, France). DMSO was obtained from Acros Organics (Geel, Belgium). Deionized water was obtained from Fresenius (Sevres, France). Drug-free sheep plasma was obtained from the CR2i investigation unit (INRA, Jouy en Josas, France).

2.2. Instrumentation and chromatographic conditions

The liquid chromatography equipment consisted of a Dionex ASI 100 T model coupled with a fluorescence detector (RF 2000). The chromatographic separation was achieved on a Wakosil II C18 RS (250 mm × 4.5 mm, 5 μm, SGE, France) analytical column protected by a C18 Wakosil precolumn (4.0 mm × 4.0 mm, 5 μm). The mobile phase consisted of 36 mM sodium dihydrogen phosphate dehydrate and 4 mM sodium 1 heptane sulfonate–acetonitrile (72:28). The pH of the mobile phase was adjusted to 3 with 1 M orthophosphoric acid. The flow used was 1.45 mL/min and the column temperature was set at 27 °C. Fluorescence detector excitation and emission wavelength were set at 355 nm and 515 nm, respectively.

2.3. Stock and standard solutions

Individual stock solutions of SN38 (0.5 mg/mL) and IS (1 mg/mL) were prepared in DMSO. After preparation, the stock solution was divided into aliquots and stored at −20 °C. CPT11 (Campto® solution 20 mg/mL) was stored at 4 °C after opening [22,23].

Separate stock solutions were prepared for the calibration curve samples and quality controls. Solutions were obtained by serial dilutions of CPT11 and SN38 stock solutions with the mobile phase. Calibration standards and quality controls were prepared by spiking 100 μL of drug-free sheep plasma with 100 μL of these solutions. Plasma calibration standards were in the range of 5–5000 and 5–240 ng/mL for CP11 and SN38, respectively. Quality control concentrations were 10, 100 and 3500 ng/mL for CPT11, 20 and 150 ng/mL for SN38.

2.4. Sample preparation

Plasma samples were thawed at room temperature and 100 μL of IS solution was added to 100 μL of plasma. Samples were vortex-mixed for 10 s. 400 μL of acetonitrile was added for protein precipitation. Centrifugation was performed at 3000 × *g* for 5 min. 100 μL of HCl (1 M) was added to 100 μL of the supernatant for the conversion of carboxylate to lactone forms. 200 μL of this mixture was injected into the LC system.

2.5. Method validation

2.5.1. Selectivity

The selectivity of the method was assessed by analyzing 9 independent blank plasma samples obtained from drug-free sheep.

2.5.2. Extraction recovery

The extraction recovery of CPT11 and SN38 from plasma was determined at several concentrations: 10, 100 and 3500 ng/mL for CPT11; 20 and 150 ng/mL for SN38. Samples ($n = 6$ per day and per concentration over 3 days) were analyzed and the resultant peak areas were compared to those obtained from direct injection of standard solutions of equivalent concentrations. The recovery of IS was determined similarly.

2.5.3. Linearity

The linearity of the method was assessed using the calibration curves ($n = 3$) in the range of 5–5000 and 5–240 ng/mL for CPT11 and SN38, respectively. Calibration curves were constructed by plotting peak area ratios of CPT11 or SN38 to IS against concentration with a weight of $1/\chi$.

2.5.4. Accuracy and precision

The intra- and inter-day accuracy and precision were evaluated by the analysis of LLOQ, low and high quality control (QC) samples ($n = 6$ samples per concentration) on the same day, over 3 days. The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy was calculated as the relative error (%RE) from the respective nominal concentration. The precision was estimated as percentage relative standard deviation (%RSD).

2.5.5. Stability

To determine the stability of samples stored at room temperature over 24 h, additional sets of samples were stored at ambient temperature. All determinations were conducted in triplicate.

2.6. Application of the method

2.6.1. Pharmacokinetic study after PACE in sheep model

The proposed method was applied to measure the plasmatic release of CPT11 from DEB after PACE on 28 adult sheep, weighing between 70 and 85 kg. Animal housing, handling and experimentation were performed in accordance with the guidelines issued by the European Economic Community (Journal Officiel de la Commmunauté Européenne, 18/12/86-authorization L3600; animal house licence number A78716). 24 sheep were embolized with 2 mL of 300–500 μm DEB (DC Bead™ Biocompatibles Ltd., UK) preloaded with 0, 20, 50 or 100 mg (DEB maximum capacity). DEB–CPT11 were delivered into the left pulmonary artery by selective catheterisation, under fluoroscopic guidance. In the control group ($n = 4$), CPT11 was directly injected in the pulmonary artery without any vector (50 and 100 mg). Sheep sacrifice was performed after 4 days or 4 weeks. Blood was sampled at various times up to sacrifice. Blood samples were collected on heparin and centrifuged at $3000 \times g$ for 10 min. Plasma was stored at −20 °C until analysis. Pharmacokinetic parameters: AUCs of average profiles, C_{max} (maximum plasma concentration) and $t_{½}$ (plasma terminal half-life) were used for comparisons between groups. They were determined using WinNonLin® software (v 4.1). Data were compared using a Mann–Whitney test (MW) (Statview®, v 5).

2.6.2. Application to sheep lymph dosage

We applied this method to sheep lymph and proceed to the same validation as in plasma (selectivity, extraction recovery, linearity, accuracy and precision).

2.6.3. Application to human plasma dosage

This method was applied to the determination of CPT11 and SN38 in plasma from one patient who received an intravenous infusion of CPT11.

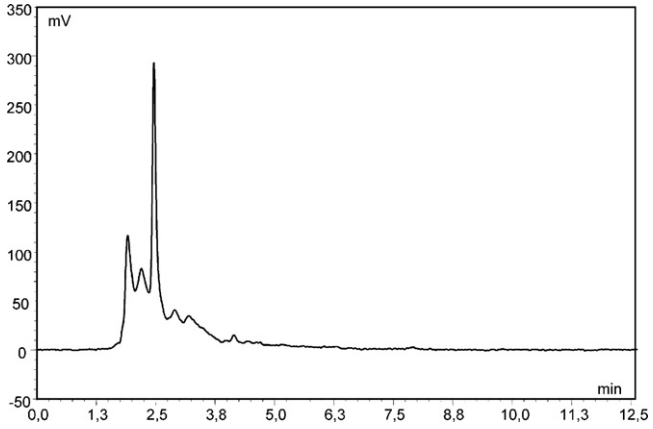

Fig. 2. Chromatogram of blank sheep plasma (for chromatographic conditions, see text).

3. Results and discussion

3.1. Liquid chromatography method

Lactone forms of CPT11 and SN38 were unstable after storage at room temperature [17], requiring a rapid freezing of biological samples after blood collection to prevent continued hydrolysis to the carboxylate forms [24]. Owing to limited clinical applicability of methods based on estimation of lactone levels only, total (lactone plus carboxylate forms) concentrations of CPT11 and SN38 were measured in the present study. Moreover, the monitoring of total CPT11 and SN38 has essentially the same clinical significance as the monitoring of lactone CPT11 and SN38 [25].

Fluorescence detector operated at excitation and emission wavelengths of 355 nm and 515 nm, respectively, which yielded the optimum signal-to-noise ratio for all compounds. The composition and the pH of our mobile phase were selected in order to optimize separation factors and peak shapes of the analytes. The total run time was 13 min. The retention times of CPT11, SN38 and CPT were respectively 4.7, 6.5 and 8 min.

3.2. Sample preparation, selectivity and extraction recovery

We used a simple one-step protein precipitation–extraction with acetonitrile in order to minimize base-line distortion and optimize extraction recovery. As shown in Figs. 2 and 3, analysis of plasma samples prepared using extraction with acetonitrile showed no peak interferences with CPT11, SN38 and IS. The mean extraction recoveries for CPT11 in plasma were 96 ± 6% at

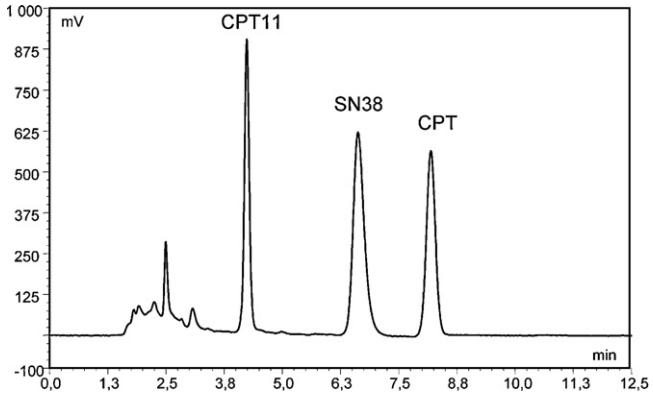

Fig. 3. Chromatogram of plasma spiked with CPT11 (100 ng/mL), SN38 (150 ng/mL) and CPT (65 ng/mL) (for chromatographic conditions, see text).

Table 1
Intra-day ($n=6$) and inter-day ($n=3$) precision and accuracy for CPT11 and SN38 analysis.

		Quality control samples (ng/mL)					
		CPT11				SN38	
		Range 1		Range 2		20	150
		10	100	100	3500		
Accuracy	Mean (%RE)	96.4	104.2	106.1	102.5	96.6	100.4
Precision	Intra-day (%RSD)	5.6	1.6	1.1	0.7	1.6	0.8
	Inter-day (%RSD)	11.1	2.6	3.6	2.9	3.9	5.0

10 ng/mL, 99 ± 2% at 100 ng/mL and 99.7 ± 0.3% at 3500 ng/mL and for SN38, 96 ± 4% at 20 ng/mL and 97 ± 5% at 150 ng/mL. The mean recovery for IS from plasma was 99.0 ± 0.4%. These results suggest that there is no relevant difference in extraction recovery at different concentrations of CPT11 and SN38 in plasma samples. Our precipitation–extraction method is cheap, simple and has very good extraction recoveries when compared to the previously reported solid-phase extraction methods [15,16].

Acidification of plasma by the addition of HCl (1 M) was achieved in order to shift the equilibrium hydrolysis reaction between the two drug forms (lactone and carboxylate) towards the lactone form.

3.3. Linearity and lower limit of quantification

Because of the large range of concentrations studied (5–5000 ng/mL), two calibration curves were necessary for the quantification of CPT11: one for low (5–100 ng/mL) and the other for high concentrations (100–5000 ng/mL). Both calibration curves were linear in the studied range. Representative linear equations for CPT11 in plasma were $y = 2.1305x + 4.2509$ in the range of 5–100 ng/mL and $y = 0.0447x - 0.2742$ in the range of 100–5000 ng/mL. For SN38 in plasma, the linear equation was $y = 0.8613x + 0.3165$ in the range of 5–240 ng/mL. Coefficients of correlation (r^2) for CPT11 and SN38 in plasma were ≥0.998.

CPT11 and SN38 LLOQ was 5 ng/mL in plasma for an accuracy between 85 and 115% and a RSD ≤ 15%. This value is equivalent or slightly higher than those reported in some publications [17,18,21,26] that validated their methods with an accuracy between 80 and 120% and a RSD ≤ 20%. Within these limits, our LLOQ would be 2 ng/mL. Improvements in sensitivity could be achieved by increasing the gain of fluorescence detector. However, the sensitivity of our method is acceptable for our preclinical study goal.

3.4. Accuracy and precision

For acceptable intra- and inter-day values, accuracy should be within 85–115% and RSD values should be ≤15% over the calibration range. Our method showed good accuracy and precision in plasma samples. The results are presented in Table 1.

3.5. Stability

The results of stability experiments showed that no significant degradation occurred at ambient temperature within 24 h, allowing samples to be prepared during the day with consecutive analysis overnight. All the RSD values were under 10%.

3.6. Application of the method

3.6.1. Pharmacokinetic study after PACE in sheep model

CPT11 eluted from DEB appeared rapidly in plasma after embolization and T_{max} was within the first 5 min. C_{max} and AUC of CPT11 in control groups were significantly higher than those in embolized groups at several times ($p = 0.0036$ and $p = 0.0393$, respectively) (Fig. 4). Therefore, DEB allowed sustained CPT11 delivery. In embolized groups, C_{max} and AUC of CPT11 increased significantly with loaded CPT11 dose ($p = 0.0078$ and $p = 0.0008$, respectively) (Fig. 5). The release of CPT11 from DEB in plasma lasted several hours (4–6 h) for groups 20 and 50 mg and 24 h for group 100 mg. Half-life elimination was not significantly different between groups. SN38 was not detected in any sheep plasma sam-

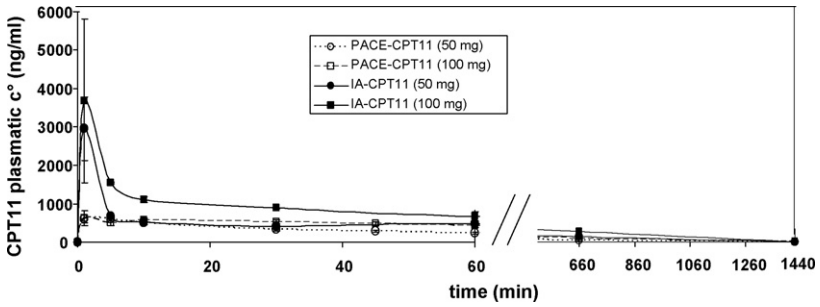

Fig. 4. Mean plasma concentration–time curves of CPT11 after PACE versus direct intra-arterial (IA) injection ($n = 16$ animals).

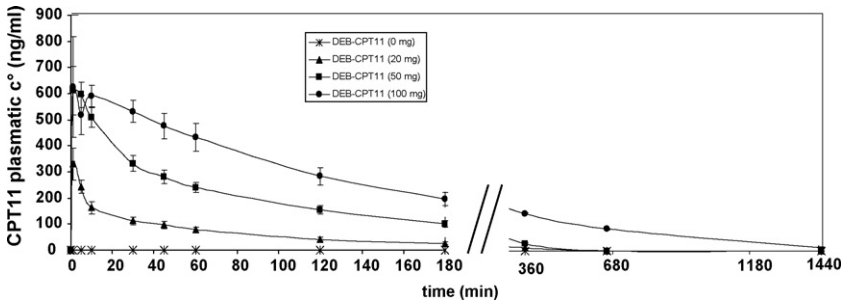

Fig. 5. Mean plasma concentration–time curves of CPT11 after PACE using 0, 20, 50 and 100 mg preloaded DEB–CPT11 ($n = 24$ animals).

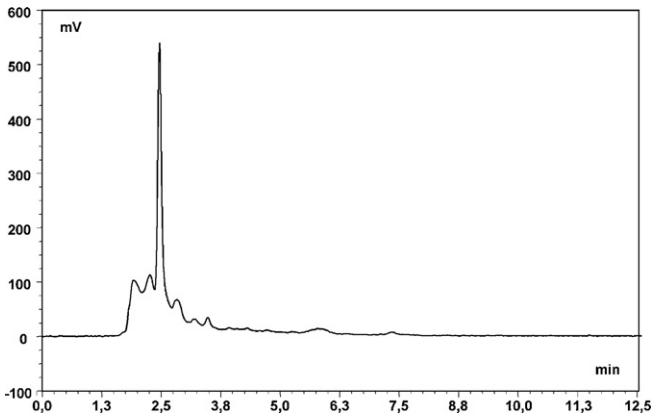

Fig. 6. Chromatogram of blank human plasma of non-treated volunteers (for chromatographic conditions, see text).

ple. This result can be explained by the absence [27] or the very low activity in sheep plasma of the carboxylesterase enzyme responsible for CPT11 activation to SN38. Koizumi et al. reported less than 5 ng/mL for SN38 C_{max} after intravenous administration of CPT11 (100 mg) to sheep weighing 28–32 kg [28].

3.6.2. Application to sheep lymph dosage

The method was successfully applied to sheep lymph for the determination of CPT11. The extraction recoveries were ≥99%. Calibration curves were linear over a range of 5–3500 ng/mL and correlation coefficients were greater than 0.999. The accuracy was ≥95% and the intra- and inter-day precision was ≤10%. The LLOQ was 5 ng/mL. This assay will be used for the second part of our study on CPT11 distribution in lymph and plasma after PACE using DEB–CPT11 versus IV administration.

3.6.3. Application to human plasma dosage

The analysis of blank plasmas of non-treated volunteers showed no matrix effect on CPT11, SN38, CPT analysis (Figs. 3 and 6). Our method was applied to determine CPT11 and SN38 concentrations in plasma following a 90 min infusion of CPT11 (100 mg/m^2) to one patient. CPT11 and SN38 concentrations 30 min after the end of infusion were respectively 2656 and 22 ng/mL. Hence, our method can be easily applied to human plasma to determine CPT11 and SN38 plasmatic concentrations.

4. Conclusion

This paper reports the first analysis of CPT11 and SN38 in sheep plasma. This method is rapid, simple and not expensive. Moreover, it is sensitive, specific, accurate, reproducible and offers significant advantages over the existing methods in terms of chromatographic conditions and sample preparation. Furthermore, our method can also be easily applied to sheep lymph or human plasma analysis and it was successfully applied to a pharmacokinetic study on plasmatic release of CPT11 from DEB after PACE in a sheep model.

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