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Journal of Chromatography B, 740 (2000) 159–168

JOURNAL OF CHROMATOGRAPHY B

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Simple and rapid determination of irinotecan and its metabolite SN-38 in plasma by high-performance liquid-chromatography: application to clinical pharmacokinetic studies

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Received 10 June 1999; received in revised form 23 November 1999; accepted 24 January 2000

Abstract

Irinotecan (CPT-11) is an anticancer agent widely employed in the treatment of colorectal carcinoma. A simple, rapid and sensitive high-performance liquid chromatographic method for the simultaneous determination of CPT-11 and its metabolite SN-38 in plasma, and their preliminary clinical pharmacokinetics are described. Both deproteinisation of plasma specimens (100 μl) and addition of the internal standard, camptothecin (CPT), are achieved by incorporating to samples 100 μl of a solution of CPT (1 μg/ml) in acetonitrile–1 m*M* orthophosphoric acid (90:10); 200 μl of this acidified acetonitrile solution, drug-free, is also added to accomplish complete deproteinisation: this procedure reduces sample preparation time to a minimum. After deproteinisation, samples are treated with potassium dihydrogenphosphate (0.1 *M*) and injected into a Nucleosil C_{18} (5 μm, 250×4.0 mm) column. Mobile phase consists of potassium dihydrogenphosphate (0.1 *M*)–acetonitrile (67:33), at a flow-rate of 1 ml/min. CPT-11, SN-38 and CPT are detected by fluorescence with excitation wavelength set at 228 nm and emission wavelengths of CPT-11, SN-38 and CPT fixed, respectively, at 450, 543 and 433 nm. The limits of quantitation for CPT-11 and SN-38 are 1.0 and 0.5 ng/ml, respectively. This method shows good precision: the within day relative standard deviation (RSD) for CPT-11 (1–10 000 ng/ml) is 5.17% (range 2.15–8.27%) and for SN-38 (0.5–400 ng/ml) is 4.33% (1.32–7.78%); the between-day RSDs for CPT-11 and SN-38, in the previously described ranges, are 6.82% (5.03–10.8%) and 4.94% (2.09–9.30%), respectively. Using this assay, plasma pharmacokinetics of CPT-11, SN-38 and its glucuronidated form, SN-38G, have been determined in one patient receiving 200 mg/m^2 of CPT-11 as a 90 min intravenous infusion. The peak plasma concentration of CPT-11 at the end of the infusion is 3800 ng/ml. Plasma decay is biphasic with a terminal half-life of 11.6 h. The volume of distribution at steady state (V_{ss}) is 203 l/m^2, and the total body clearance (Cl) is 14.8 l/h·m^2. The maximum concentrations of SN-38 and SN-38G reach 28.9 and 151 ng/ml, respectively.

Keywords: HPLC; Pharmacokinetics; Irinotecan; SN-38

1. Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; CPT-11) (Fig. 1) is a semisynthetic water-soluble camptothecin produced in an attempt to reduce the toxicity and improve the therapeutic efficacy of the parent compound camptothecin (CPT). CPT-11 inhibits topoisomerase I activity through the formation of stable topoisomerase I-DNA cleavable complexes [1–3]. CPT-11 acts

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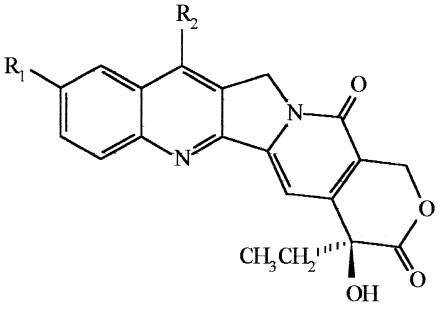
PII: S0378-4347(00)00048-7



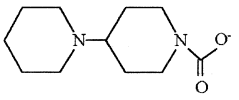
Compound	Substituent group R_1	R_2
CPT-11		$-CH_2-CH_3$
SN-38	OH	$-CH_2-CH_3$
CPT	H	H

Fig. 1. Structures of CPT-11, its active metabolite SN-38 and the parent drug camptothecin (CPT).

as a prodrug in vivo and is converted to its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) by the enzyme carboxyl esterase [4]. SN-38 has been shown to undergo glucuronic acid conjugation to form the corresponding glucuronide (SN-38G) which is the major elimination pathway of SN-38 [5–8]. Several phase I trials have efficiently explored the pharmacokinetics of CPT-11 and SN-38 in patients receiving CPT-11 therapy [5,7–18]. However, there is less information describing the clinical pharmacokinetics of SN-38G [5–8,14,16]. Insufficient conjugation of SN-38 has been recently related to the incidence of dose-limiting intestinal toxicity [5,8], which reveals the importance of determining SN-38G concentrations.

Numerous high-performance liquid chromatographic (HPLC) methods have been developed for the determination of camptothecin derivatives in buffered solutions [19] and in biological fluids [19–28]. Sample pretreatment procedures vary greatly and most of them involve several phases such as addition of internal standard (I.S.) and deproteination of plasma by means of a precipitating agent. Moreover some of the published methods include long and tedious liquid–liquid extraction procedures [22,28] or solid-phase extraction processes with C_{18} columns [24,27]. Every method also incorporates at least one acidification or alkalinization step.

Analytical procedures determining camptothecin derivatives could be classified in two groups: methods quantifying total concentration of these drugs [20,22–24,27,28] and methods that discriminate between the lactone and carboxylate forms [19,21,22,25,26]. Since lactone is the active form of these anticancer agents, discriminating between lactone and carboxylate forms appears to be an aim. However, clinical applicability of this kind of assays is hampered by stability problems associated with lactone–carboxylate interconversion [22]. Moreover, for practical purposes, monitoring of total CPT-11 and SN-38 has essentially the same clinical significance as monitoring of lactone CPT-11 and SN-38 [29].

The method presented here determines total concentration of CPT-11 and SN-38 in plasma. By means of the incorporation of I.S. (CPT) to plasma samples in acidified acetonitrile solution, we condense in just one phase three common operations any other method develops separately: addition of I.S., acidification of plasma and protein precipitation; so that the assay gains in simplicity and rapidity. Besides, the method becomes precise and accurate and at least as sensitive as other assays determining simultaneously CPT-11 and SN-38 [19–25].

Using this assay we have quantified plasma concentrations of CPT-11, SN-38 and SN-38G. We describe here the assay methodology, and report preliminary clinical pharmacokinetic results obtained after the administration of a 90 min intravenous infusion of CPT-11 (200 mg/m^2) to a representative patient.

2. Experimental

2.1. Reagents

CPT-11, SN-38 and CPT were kindly supplied by Rhône–Poulenc Rorer (Centre de Recherche de

Vitry-Alfortville, France). HPLC-grade acetonitrile and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany) and analytical grade orthophosphoric acid was obtained from Panreac (Barcelona, Spain). β-Glucuronidase was acquired from Sigma-Aldrich (Steinheim, Germany).

For stock solutions, CPT-11, SN-38 and CPT are dissolved in acetonitrile–1 m*M* orthophosphoric acid (90:10, pH~3) and stored at 4°C in darkness.

2.2. Sample preparation

Blood samples are collected on heparin and immediately centrifuged at 3000 *g* for 5 min. Plasma is stored at −30°C until analysis. Each sample is allowed to thaw at room temperature, and 100 μl of a solution of CPT (I.S.) (1 μg/ml) and 200 μl of a mixture of acetonitrile–1 m*M* orthophosphoric acid (90:10) is added to 100 μl of plasma. The tube is vortex-mixed for 10 s, centrifuged at 4000 *g* for 5 min and 600 μl of 0.1 *M* potassium dihydrogenphosphate (pH 4.2) is added to the supernatant. Then, 100 μl of this mixture is injected into the HPLC system.

Standards are prepared from normal human plasma spiked with different amounts of CPT-11 and SN-38, both in acetonitrile–1 m*M* orthophosphoric acid (90:10) solution, together with the corresponding I.S. solution: 100 μl of each of the three solutions (CPT-11, SN-38 and CPT) are added to 100 μl of plasma. The standards are then analysed as patient samples.

For SN-38G estimation, plasma samples are analysed as described above. Prior to deproteinisation, the samples are incubated with 1000 *u* of β-glucuronidase for 2 h at 37°C [8,14,30].

2.3. High-performance liquid chromatography

The HPLC equipment consists of a HP 1100 model with a fluorescence detector (1046 A model). Separation of compounds is achieved using a Nucleosil RP-18 (5 μm, 250×4.0 mm) analytical column protected by a C_{18} Nucleosil precolumn (5 μm, 4.0×4.0 mm). The mobile phase is 0.1 *M* potassium dihydrogenphosphate (adjusted to pH 4.2 with 1 *M* HCl)–acetonitrile (67:33); the flow used is 1 ml/min and the column temperature is set at 30°C. The fluorescence detector excitation wavelength is set at 228 nm and the emission wavelengths of CPT-11, SN-38 and CPT are, respectively, 450, 543 and 433 nm.

2.4. Recovery

The recovery from plasma is determined by comparing the areas of pure standards with those of extracted plasma samples containing the same amount of standards.

2.5. Determination of CPT-11, SN-38 and SN-38G in plasma

The concentrations of CPT-11 and SN-38 are determined from area ratios of either CPT-11 or SN-38 to the I.S. (CPT), by reference to the calibration graphs obtained, respectively, for CPT-11 in the concentration range 1–10 000 ng/ml and for SN-38 in the concentration range 0.5–400 ng/ml. Linear least squares regression is done by weighting a reciprocal of the concentration spike squared.

SN-38G concentrations, as it is explained in Section 2.2., are determined as the increase in SN-38 concentrations following incubation with β-glucuronidase.

2.6. Determination of precision, accuracy and lower limit of quantitation

Five replicates with six different concentrations of either CPT-11, ranging from 1 to 10 000 ng/ml, or SN-38, from 0.5 to 400 ng/ml, in human plasma are processed as described above to determine the relative standard deviation of the mean (RSD) and relative mean error (RME) using the following equations: RSD=(SD/mean)·100; RME=[(mean−SCON)/SCON]·100, where SD is the standard deviation of the mean and SCON is the spiked concentration of the analyte.

The RSD and RME are indicators of precision and accuracy, respectively. The lower limit of quantitation (LOQ) for each analyte is calculated as the lowest standard concentration with RSD and RME< 20%.

2.7. Application to clinical pharmacokinetic studies

This assay has been utilised to determine CPT-11, SN-38 and SN-38G concentrations in plasma follow-

ing a 90 min infusion of the drug (200 mg/m^2) to one patient. Blood samples were obtained at 30 and 60 min during infusion, at the end of infusion (90 min), and 5, 15, 30 min and 1, 2, 4, 6, 12, 24, 48 and 72 h after the end of the infusion.

Pharmacokinetic parameters of CPT-11 have been calculated using standard equations [31]. The following parameters are determined: half-life as $0.693/\lambda_i$, where λ_i is the slope of the terminal phase; AUC is estimated by the trapezoidal method with extrapolation to infinity ($AUC = AUC_{0-72h} + C_{72h}/\lambda_i$); the volume of distribution at steady state (V_{ss}) is determined as $V_{ss} = Dose \cdot AUMC/AUC^2$, where AUMC is the area under the moment curve with extrapolation to infinity ($AUMC = AUMC_{0-72h} + C_{72h}/\lambda_i^2$); and the total plasma clearance (Cl) is calculated as Dose/AUC. The concentration at the end of the i.v. infusion (C_{max}) is the actual plasma concentration assayed. For SN-38 and SN-38G the following parameters are estimated: the maximum concentration achieved (C_{max}), AUC calculated as described above and time to reach peak plasma level (T_{max}).

3. Results and discussion

3.1. HPLC assay development

3.1.1. Fluorescence wavelengths selection

Fluorescence is selected as CPT-11, SN-38 and CPT (I.S.) show native fluorescence, very convenient for sensitive and selective determination. Fig. 2 presents the excitation and emission spectra of CPT-11, SN-38 and CPT. As this figure shows, the emission (228 nm) and excitation wavelengths (450, 543 and 433 nm for CPT-11, SN-38 and CPT, respectively) selected for the determination of these drugs are the optimum ones for each compound in order to allow maximum response.

3.1.2. Sample treatment

In the determination of camptothecin derivatives, three procedures are usually performed: addition of I.S., acidification of plasma (when determining total drug concentration) and deproteinisation of plasma samples.

I.S., usually CPT, is added as a solution [22,23,27,28] or as an evaporated residue [21]. In addition, acidification of plasma is achieved in order to shift the equilibrium hydrolysis reaction between the two drug forms (lactone and carboxylate) towards the lactone form. On the other hand, several precipitating agents have been tested such as methanol [19,23,25], acetonitrile [28], zinc sulphate [26] or different solutions containing methanol: acetonitrile–methanol (1:1) [21], perchloric acid–methanol (1:1) [20] and perchloric acid–methanol–water (1:20:20) [22].

Since CPT is soluble in acidified acetonitrile, we have successfully tested its incorporation to plasma samples by means of the addition of 100 μl of a 1 μg/ml solution of I.S. in acetonitrile–1 m*M* orthophosphoric acid (90:10, pH~3). Hence, we have concentrated in just one those three mentioned procedures, which are carried out separately by any other assay, and therefore the method becomes much simpler and rapid. Finally, and in order to accomplish complete deproteinisation, 200 μl of acidified acetonitrile solution is also added.

Considering that CPT-11 and SN-38 can also be dissolved in acidified acetonitrile, standards are prepared by means of spiking free-drug plasma with 100 μl of each one of these three solutions: CPT-11, SN-38 and CPT. Therefore, standards and patient samples undergo the same procedure (300 μl of acetonitrile solution is added to both of them).

3.1.3. Addition of buffer to the precipitated samples

The addition of 0.1 *M* potassium dihydrogenphosphate (pH 4.2) to the precipitated samples results in the improvement of the quality of the peaks. As shown in Table 1, the best outcome is obtained by the incorporation of either 600 or 800 μl of buffer to the samples. We selected the lower volume (600 μl) in order to avoid an excessive dilution of the samples and, hence, improve sensitivity.

3.1.4. Stability of injection samples

Processed plasma samples are found to be stable at

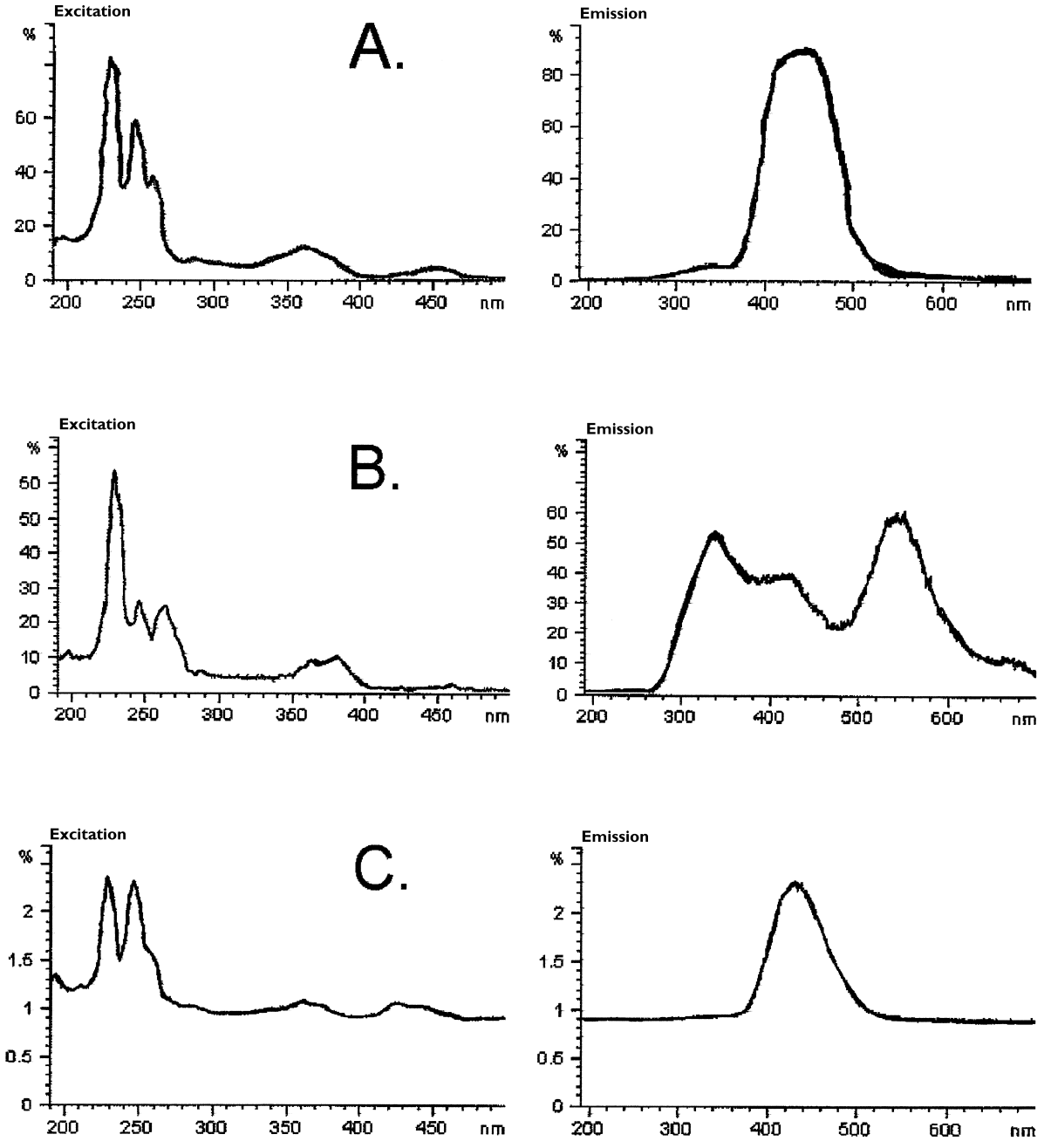


Fig. 2. Excitation and emission spectra of (A) CPT-11, (B) SN-38 and (C) CPT.

room temperature upon standing in the autosampler tray for at least 24 h, allowing samples to be prepared during the day with consecutive analysis overnight.

3.2. Assay validation

The analytical methodology has been validated in terms of selectivity, recovery, linearity, limit of

Table 1
CPT-11, SN-38 and CPT (I.S.) peak shapes according to the volume of 0.1 *M* potassium dihydrogenphosphate (pH 4.2) solution added to samples

Volume of 0.1 *M* potassium dihydrogenphosphate (μl)	Peak width			Peak symmetry		
	CPT-11	SN-38	CPT	CPT-11	SN-38	CPT
0	1.72	1.52	1.95	0.43	0.52	1.74
200	0.73	0.68	0.88	0.61	0.68	1.38
400	0.35	0.41	0.47	0.78	0.85	1.25
600	**0.19**	**0.18**	**0.22**	**0.88**	**0.95**	**1.12**
800	0.19	0.19	0.21	0.91	1.10	0.95

quantitation, precision and accuracy for both CPT-11 and SN-38.

3.2.1. Selectivity

No interfering peaks are observed in any of the plasma pools used in our studies, or in plasma samples obtained from patients who have not received prior administration of CPT-11 (Fig. 3A). A representative chromatogram of a human plasma extract after administration of CPT-11 is shown in Fig. 3B. Resolution of the compounds of interest is optimum with a mobile phase composition of 0.1 *M* potassium dihydrogenphosphate (pH 4.2)–acetonitrile (67:33).

3.2.2. Recovery

The overall mean recoveries of CPT-11 and SN-38 are 93.5±4.11 and 93.1±4.35, respectively (Table 2). The recovery of CPT (I.S.) measured at the concentration studied (1 μg/ml) is 93.8±3.11 ($n=5$). As it is shown in Table 2, recoveries for both CPT-11 and SN-38 are not only high, but also similar in all concentrations studied.

3.2.3. Linearity

Because of both the type of fluorescence detector used, which scaled fluorescence in percentage, and the large range of concentrations studied (1–10 000 ng/ml), two calibration graphs are, in this case, necessary for the quantitation of CPT-11: one for

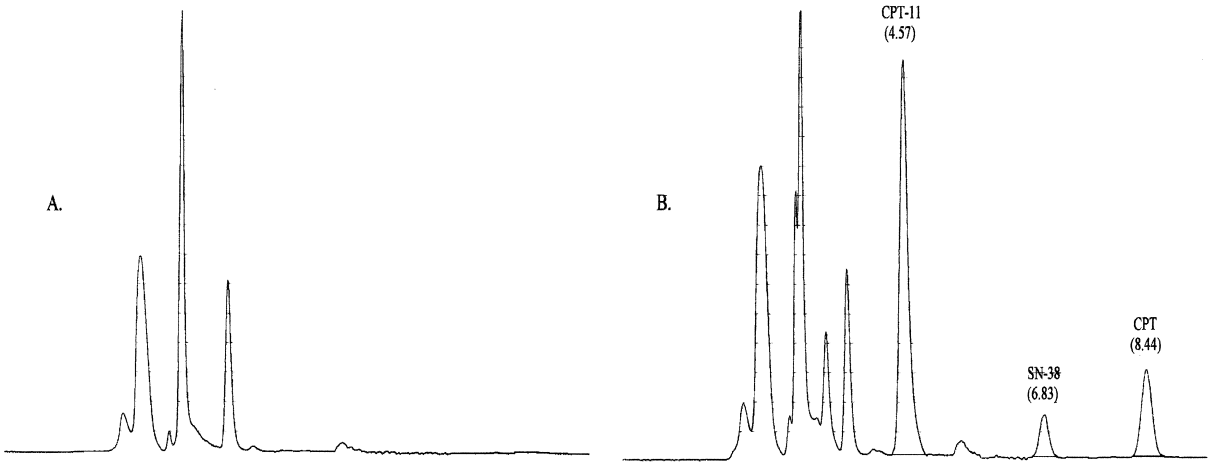


Fig. 3. HPLC of: (A) a predose plasma extract, and (B) a human plasma extract after administration of CPT-11. Peaks: CPT-11 (4.57 min), SN-38 (6.83 min) and internal standard CPT (8.44 min).

Table 2
Recoveries of CPT-11 and SN-38 from human plasma ($n=5$); results are expressed as mean±SD (standard deviation)

CPT-11		SN-38	
Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)
1	90.0±6.80	0.5	92.7±5.25
10	95.5±2.00	2	92.6±3.04
50	93.2±3.25	5	94.9±6.25
500	94.6±2.52	20	91.2±2.12
2000	95.0±3.28	100	96.7±2.28
10 000	92.8±4.40	400	90.8±3.91

low and the other one for high concentrations. The amplification factor of the detector changes from low concentrations (PMTgain=12) to high concentrations (PMTgain=7) while the rest of the method remains unchanged. The detection program is detailed in Table 3.

The calibration graphs for CPT-11 are linear in both concentration ranges assayed: from 1 to 500 ng/ml and from 500 to 10 000 ng/ml; likewise, the calibration graph for SN-38 is linear from 0.5 to 400 ng/ml. The means±SD of the calibration graphs obtained for low and high concentrations of CPT-11 yield the following equations: $y=(0.03504\pm0.00045)x+(0.00296\pm0.00268)$ and $y=(0.00141\pm0.00005)x+(-0.03660\pm0.01838)$, respectively. The analogous equation for SN-38 in the concentration range assayed is $y=(0.17052\pm0.00231)x+(-0.00283\pm0.00331)$. In all these equations, y is the area ratio of the peak area of either CPT-11 or SN-38 to that of CPT (IS) and x is the concentration of CPT-11 or SN-38.

As described at point 2.5., SN-38G concentrations are calculated as the increase in SN-38 concentrations following incubation with β-glucuronidase. This indirect determination is justified since resulting levels are within ±20% of the nominal values.

3.2.4. Lower limit of quantitation

The LOQ is 1.0 ng/ml for CPT-11 and 0.5 ng/ml for SN-38, which are in accordance with the most sensitive assays determining both drugs [22,23]. Using this method, it is possible to determine plasma concentrations of CPT-11 and SN-38 from samples obtained 72 h after the administration of a 90 min infusion of a 200 mg/m^2 dose of CPT-11.

3.2.5. Precision and accuracy

Tables 4 and 5 show, respectively, the within-day and between-day precision (RSD) and accuracy

Table 3
Detection program of the HPLC determination of camptothecin derivatives

Time range (min)	Compound detected	Concentration (ng/ml)	PMTgain	λ_{ex} (nm)	λ_{em} (nm)
0.00–5.80	CPT-11	1–500	12	228	450
		500–10 000	7		
5.80–7.90	SN-38	0.5–400	16	228	543
7.90–9.50	CPT (I.S.)	1000	6	228	433

Table 4
Within-day reproducibility of the HPLC determination of CPT-11 and SN-38 in human plasma ($n=5$).

CPT-11			SN-38		
Concentration (ng/ml)	RSD (%)	RME (%)	Concentration (ng/ml)	RSD (%)	RME (%)
1	8.27	−8.50	0.5	7.78	10.27
10	4.84	−7.06	2	5.88	3.99
50	6.36	5.48	5	2.94	−1.22
500	5.28	5.92	20	4.05	0.58
2000	4.14	2.82	100	3.99	−2.12
10 000	2.15	−1.59	400	1.32	−1.08

Table 5
Between-day reproducibility of the HPLC determination of CPT-11 and SN-38 in human plasma ($n=5$)

CPT-11			SN-38		
Concentration (ng/ml)	RSD (%)	RME (%)	Concentration (ng/ml)	RSD (%)	RME (%)
1	10.80	−8.20	0.5	9.30	6.10
10	7.29	−9.10	2	4.65	11.30
50	6.99	6.44	5	4.15	6.18
500	5.03	2.34	20	5.66	−5.06
2000	5.22	4.16	100	3.78	4.33
10 000	5.56	1.62	400	2.09	−4.85

(RME) for this assay. As can be seen in these tables, precision and accuracy of this method are acceptable and hardly depend on the concentration assayed or on the day of the assay.

3.3. Clinical pharmacokinetics of CPT-11, SN-38 and SN-38G

Plasma concentration profiles of CPT-11, SN-38 and SN-38G in one patient receiving 200 mg/m^2 of CPT-11 as a 90 min infusion are shown in Fig. 4. A biphasic plasma decay of CPT-11 is observed and, in agreement with recently reported studies [8,20], terminal half-life calculated is 11.6 h. The volume of distribution (V_{ss}) is 203 l/m^2 and the total body clearance (Cl) is 14.8 l/h·m^2, which is within the same range as described for this compound by different authors [5,8,9,13–18,24]. At this dose level, AUC_{CPT-11} is 13 480 ng·h/ml (21.6 μM·h). SN-38 and SN-38G attain peak plasma concentrations of 28.9 ng/ml (0.074 μM) and 151 ng/ml (0.266 μM), respectively; total AUC_{SN-38} is 225 ng·h/ml (0.572 μM·h) and AUC_{SN-38G} reaches 1704 ng·h/ml (3.0 μM·h). Peak concentration of SN-38 is achieved at

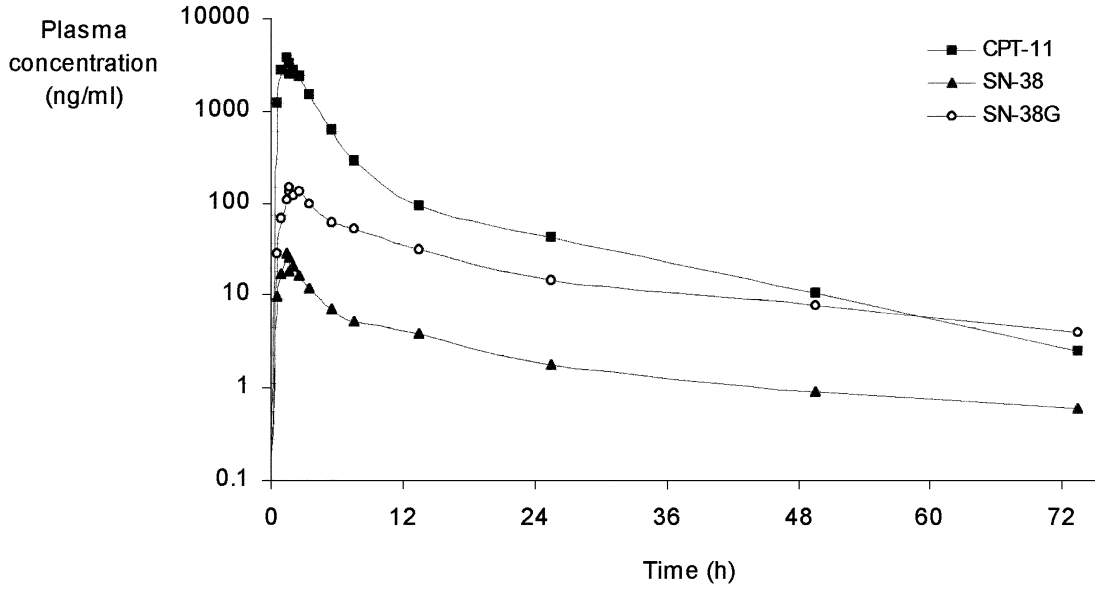


Fig. 4. Representative plasma concentrations of CPT-11, SN-38 and SN-38G in one patient after the administration of CPT-11 at a dose level of 200 mg/m^2 as a 90 min infusion. Plasma concentrations of these compounds have been determined by HPLC with fluorescence detection.

the end of the 90 min infusion of CPT-11, while peak concentration of SN-38G is obtained 15 min after the end of the infusion.

As it can be noticed, plasma SN-38G concentrations are significantly higher compared to those of SN-38 ($AUC_{SN\text{-}38G}$, expressed in $\mu M \cdot h$, is about 5 times higher than $AUC_{SN\text{-}38}$ in this patient), which indicates that, in this patient, glucuronidation is a prominent clearance mechanism for SN-38, which agrees with previously reported data [5–8].

4. Conclusions

In addition to its specificity, the method we describe here for the determination of camptothecin derivatives has the advantage of simplicity, since it requires the addition of just one reagent to achieve incorporation of I.S., deproteinisation and acidification, thereby shortening analysis time and also reducing solvent waste. For instance, a group of 10 to 20 samples are processed and ready for injection in about 15–20 min.

Furthermore, this assay provides high recoveries for CPT-11 and SN-38 and satisfactory values for precision and accuracy, and becomes one of the most sensitive methods for the simultaneous quantification of these drugs.

Acknowledgements

The authors wish to thank the financial support of Rhône–Poulenc Rorer, S.A. that made this study possible.

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