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Simultaneous determination of the camptothecin analogue CPT-11 and its active metabolite SN-38 by high-performance liquid chromatography: application to plasma pharmacokinetic studies in cancer patients

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ABSTRACT

CPT-11 {I; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin} is a new anticancer agent currently under clinical development. A sensitive high-performance liquid chromatographic assay suitable for the simultaneous determination of I and its active metabolite SN-38 (II) in human plasma, and their preliminary clinical pharmacokinetics, are described. Plasma samples were processed using a solid-phase (C_{18}) extraction step allowing mean recoveries of I, II and the internal standard camptothecin (III) of 84, 99 and 72%, respectively. The extracts were chromatographed on a C_{18} reversed-phase column with a mobile phase composed of acetonitrile, phosphate buffer and heptanesulphonic acid, with fluorescence detection. The calibration graphs were linear over a wide range of concentrations (1 ng/ml–10 μg/ml), and the lower limit of determination was 1 ng/ml for both I and II. The method showed good precision: the within-day relative standard deviation (R.S.D.) (5–1000 ng/ml) was 13.0% (range 4.9–19.4%) for I and 12.8% (6.7–19.1%) for II; the between-day R.S.D. (5–10 000 ng/ml was 7.9% (5.4–17.5%) for I and 9.7% (3.5–15.1%) for II. Using this assay, plasma pharmacokinetics of both I and II were simultaneously determined in three patients receiving 100 mg/m^2 I as a 30-min intravenous infusion. The mean peak plasma concentration of I at the end of the intravenous infusion was 2400 ± 285 ng/ml (mean ± standard error of the mean). Plasma decay was triphasic with half-lives α, β and γ of 5.4 ± 1.8 min, 2.5 ± 0.5 h and 20.2 ± 4.6 h, respectively. The volume of distribution at steady state was 105 ± 15 l/m^2, and the total body clearance was 12.5 ± 1.9 l/h · m^2. The maximum concentrations of the active metabolite II reached 36 ± 11 ng/ml.

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INTRODUCTION

Camptothecin is a plant alkaloid obtained from *Camptotheca acuminata* [1] that has shown promising antitumour properties *in vitro* [2,3] and in animal models [1,4], but has been clinically disappointing because of its serious toxicity [5–8]. The poor water solubility of camptothecin also makes its clinical use impractical.

To overcome those problems, various analogues of camptothecin have been synthesized [9], including CPT-11 {I; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin} (Fig. 1). This water-soluble drug is active *in vitro* and *in vivo* in various experimental systems, and also against pleiotropic drug-resistant tumours [9–11]. The mechanism of action of I is presumed to be mediated through topoisomerase I inhibition [12].

Although a high-performance liquid chromatographic (HPLC) method was previously reported for the determination of I and its active metabolite SN-38 (II) in biological fluids [13], it requires two separate analyses. In an effort to decrease the analysis time and improve the accuracy by minimizing handling of biological samples we developed a sensitive HPLC assay allowing the simultaneous determination of both I and its active metabolite II. We describe here the assay methodology, and report preliminary clinical pharmacokinetic results obtained at a starting dose of 100 mg/m^2 during a phase I study exploring a single administration every three weeks.

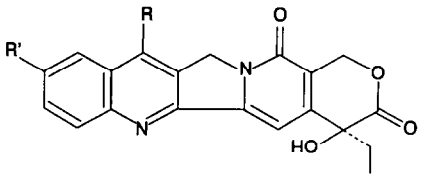

Fig. 1. Structures of CPT-11 (I), its active metabolite SN-38 (II and camptothecin (III).

EXPERIMENTAL

Chemicals

CPT-11 (I), SN-38 (II) and the internal standard camptothecin (III) (Fig. 1) were synthesized and kindly provided by Yakult Honsha (Tokyo, Japan). Solvents and reagents were of the highest purity available and were obtained from Carlo Erba (Milan, Italy).

Patients

Three patients meeting standard phase I eligibility criteria were evaluated for pharmacokinetics during three courses of administration of I. The patients signed an informed consent form.

Drug administration

Drug I was supplied as a solution ready for use, in 2- or 5-ml vials containing 40 and 100 mg, respectively. The required dose (100 mg/m^2) was further diluted in 250 ml of a 0.9% solution of sodium chloride in water and administered as a 30-min intravenous (i.v.) infusion into a peripheral vein.

Plasma collection for pharmacokinetics

Heparinized blood samples (2 ml) were collected immediately pre-dose (time zero), and at the following times thereafter: 10, 20, and 30 min during the 30-min i.v. infusion; after the infusion at 5, 10, 15, 30, 45 and 60 min; then at 2, 4, 8, 12 and 24 h post-infusion. Blood was immediately centrifuged at 2000 *g* for 15 min and the plasma was transferred to a 1.5-ml polypropylene tube and frozen at –20°C until analysis.

Preparation of standards

Stock solutions (100 μg/ml) of I and II were prepared in water and methanol, respectively. Subsequent dilutions ranging from 50 ng/ml to 10 μg/ml were prepared in 0.01 *M* HCl. Aliquots of 100 μl each of I and II standards containing various concentrations of the compounds were added to 100 μl of control plasma (final volume 300 μl). These plasma standards were then frozen at −70°C until extraction. Before extraction, these 300-μl plasma standards were spiked with

50 μl of a solution of III (1 μg/ml) used as the internal standard, and the volume was completed to 1 ml by adding 650 μl of 0.01 *M* HCl. The solution of III was prepared as follows: 1 mg of III was dissolved in 25 ml of methanol, sonicated for about 20 min at room temperature, diluted with water to 10 μg/ml and further diluted with 0.01 *M* HCl to a final concentration of 1 μg/ml.

Extraction procedure

To minimize degradation of I and II, frozen plasma was quickly thawed within 3 min by immersion in a water-bath (50°C) with shaking; this procedure ensured rapid thawing and did not cause degradation. After vortex-mixing, plasma was briefly centrifuged for 4 min at 13 000 *g*. An aliquot (100 μl) of the plasma supernatant was quickly transferred to a 10-ml glass tube, followed by successive additions of 50 μl of the internal standard solution (1 μg/ml) and 850 μl of 0.01 *M* HCl (final volume 1 ml). All the following extraction steps were automated using a Bench-Mate Workstation (Zymark, Roissy, France). Before solid-phase extraction of the plasmas, the columns (Bond Elut cartridges, 100 mg, 40 μm) (J. T. Baker, Phillipsburg, NY, USA) were conditioned by sequential washing with methanol (1 ml) and water (1 ml) at a flow-rate of 0.5 ml/s. A 1-ml volume of the above plasma preparation was applied to the preconditioned cartridges at a load flow of 0.04 ml/s and the cartridge was successively washed with water (1.5 ml) and 0.01 *M* HCl (1 ml) at a flow-rate of 0.10 ml/s. Compounds were then eluted with 1.5 ml of acidic methanol (100 μl of 13 *M* HCl in 100 ml of methanol) at a flow-rate of 0.04 ml/s. The methanol eluate was evaporated to dryness under a stream of nitrogen and the residue was dissolved with 250 μl of mobile phase and then injected into the HPLC system.

High-performance liquid chromatography

HPLC analyses were carried out using a Waters (Milford, MA, USA) workstation composed of the following: a Waters TM 840 data and chromatography control station, a Professional 380 computer, two pumps (Model 510), an automatic injector (Wisp) and a programmable fluorescence detector (Model 470). Separation of compounds I–III was achieved using a reversed-phase Nucleosil octadecylsilane (10 μm) analytical column (300 mm × 3.9 mm I.D.) (S.F.C.C., Neuilly-Plaisance, France), protected by a C_{18} Nucleosil (10 μm) precolumn (22 mm × 3.5 mm I.D.). The mobile phase (flow-rate 1 ml/min) was acetonitrile–0.1 *M* potassium dihydrogenphosphate (34:66) containing 3 m*M* sodium heptanesulphonate (adjusted to pH 4 with 1 *M* HCl). The fluorescence detector wavelengths were set at 380 nm (excitation) and 500 nm (emission), to allow maximum response for the two compounds in the same run without changing wavelengths (band widths 18 nm).

Determination of I and II in plasma

The concentrations of I and II were determined from peak-area ratios of either I or II to the internal standard (III), by reference to a calibration graph obtained daily, after the thawing of plasma standards frozen at −70°C. The frozen plasma standards were stable for at least three months. The limit of detection in plasma was defined as the concentration that gave a signal three times greater than the background noise.

Determination of recovery, precision, and accuracy

The recovery from plasma was determined by comparing the peak areas of pure standards with those of extracted plasma samples containing the same amount of standards. Ten replicates with concentrations ranging from 5 to 1000 ng/ml I and II in human plasma were processed as described above to determine the within-day reproducibility. Also ten replicates with five different concentrations (5 ng/ml–10 μg/ml) were analysed three times to estimate the between-day reproducibility. The precision of the method at each concentration was calculated as the relative standard deviation (R.S.D.). The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked concentration.

Determination of pharmacokinetic parameters

Plasma concentrations of I were best fitted to a three-compartment model with constant i.v. infusion, using a non-linear regression program (PCNONLIN; Statistical Consultant, Lexington, KY, USA). Pharmacokinetic parameters were determined using standard equations [14]. Briefly, the following parameters were calculated: half-lives, as $0.693/\lambda_i$; the area under the curve (AUC) was determined by the trapezoidal method with extrapolation to infinity; the volume of distribution at steady state (V_{dss}) was determined as V_{dss} = dose · AUMC/AUC^2, where AUMC is the area under the first-moment curve that describes the time course of the plasma concentrations; the total plasma clearance (Cl) was calculated as the dose/AUC. The concentration at the end of i.v. infusion (C_{max}) was the actual plasma concentration assayed. For metabolite II the following parameters were determined: the maximum concentration achieved (C_{max}), the AUC calculated as above and the time at the peak plasma level. Results are presented as means standard error of the mean (S.E.M.).

RESULTS

HPLC assay development

A representative chromatogram of a human plasma extract after administration of I at 100 mg/m^2 is shown in Fig. 2. Resolution of the compounds of interest was optimum with a mobile phase composition of acetonitrile–0.1 *M* potassium dihydrogenphosphate with addition of 3 m*M* sodium heptanesulphonate (pH 4). Fluorescence detection was selected as I and II show native fluorescence, very convenient for sensitive and selective quantification. Although the optimum excitation and emission wavelengths are 373 and 428 nm for I and 380 and 540 nm for II, we selected 380 nm as the excitation wavelength and 500 nm as the emission wavelength to allow maximum response for both compounds in the same run without changing wavelengths. No interfering peak was observed in any of the plasma pools used in our studies, or in any plasma samples obtained from patients who had not received prior administration of I.

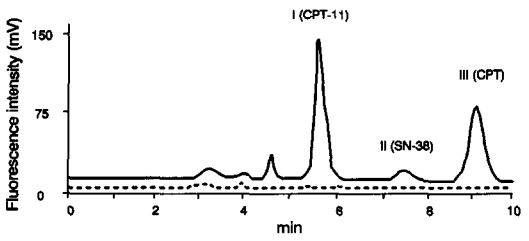

Fig. 2. HPLC of a human plasma extract (solid line) after administration of 100 mg/m^2 I (CPT-11). Peaks: I = CPT-11; II = SN-38; III = CPT, internal standard camptothecin. A predose plasma extract is shown as a dashed line. The peak at 4.6 min is an unidentified metabolite. Fluorescence detection wavelengths were set at 380 nm (excitation) and 500 nm (emission).

Validation of the assay

The analytical methodology was validated in terms of linearity, recovery, limit of determination, precision and accuracy for I and its metabolite II. The calibration graphs for both I and II in plasma were linear from 1 ng/ml to 10 μg/ml. The mean of six different calibration graphs yielded the following equations with a zero intercept: $y = 0.9513x$ ($r = 0.9997$) for I and $y = 1.024x$ ($r = 0.9958$) for II, where y is the peak-area ratio of either I or II to the internal standard III and x is the concentration of I or II. Overall mean recoveries of I, II and III were 84 ± 8, 99 ± 3 and 72 ± 4%, respectively (Table I). The limit of determination was 1 ng/ml for both compounds,

TABLE I

RECOVERIES OF CPT-11 (I), SN-38 (II) AND INTERNAL STANDARD CAMPTOTHECIN (III) FROM HUMAN PLASMA

Number of observations = 4; results are expressed as mean ± S.E.M.

Concentration (ng/ml)	Recovery (%)		
	I	II	III
5	109 ± 15	91 ± 14	84 ± 18
25	76 ± 3	102 ± 2	72 ± 3
50	75 ± 5	105 ± 4	66 ± 9
100	75 ± 4	99 ± 7	67 ± 4

TABLE II

WITHIN-DAY PRECISION AND ACCURACY OF THE HPLC DETERMINATION OF CPT-11 (I) AND SN-38 (II) IN HUMAN PLASMA

Number of observations = 10.

Concentration (ng/ml)	I		II	
	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
5	19.4	136.0	19.1	77.8
25	4.9	105.8	7.8	98.1
50	8.9	95.6	14.7	88.7
100	15.9	89.5	6.7	106.3
500	16.6	102.5	17.1	101.9
1000	12.3	91.4	11.6	96.7

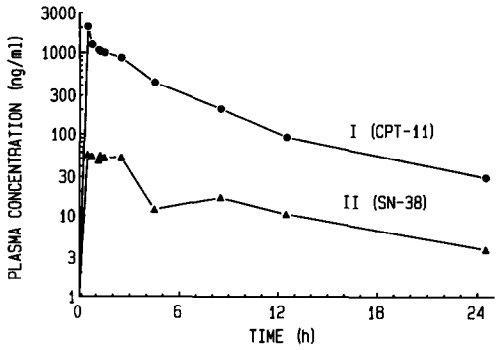

Fig. 3. Representative plasma concentration of I (CPT-11) and active metabolite II (SN-38) in one patient after the administration of I at a dose level of 100 mg/m^2 as a 30-min i.v. infusion. Plasma concentrations of both compounds were simultaneously determined by HPLC with fluorescence detection.

which was sufficient for our pharmacokinetic studies. The precision was good, with mean within-day R.S.D.s of 13.0% for I and 12.8% for II, with mean accuracies of 103.5 and 94.9%, respectively (Table II). The mean between-day R.S.D.s were 7.9% for I and 9.7% for II with accuracies of 106.4 and 94.3%, respectively (Table III).

Clinical pharmacokinetics of I and II

Plasma concentration profiles of both I and II in a patient receiving 100 mg/m^2 I as a 30-min i.v. infusion are shown in Fig. 3. Plasma decay of I was triphasic with mean half-lives α, β and γ of 5.4 ± 1.8 min, 2.5 ± 0.5 h and 20.2 ± 4.6 h, respectively (three patients). The mean volume of distribution (V_{dss}) was large, 105 ± 15 l/m^2, and the total body clearance (*Cl*) was 12.5 ± 1.9 l/h · m^2. At this dose level, the total AUC of I was 8331 ± 1192 ng h/ml. The active metabolite II attained peak plasma levels of 35.5 ± 10.9 ng/ml with a total AUC of 257 ng h/ml. Peak levels of II were achieved 30 min after the end of 30-min i.v. infusion of I.

DISCUSSION

Because of formulation and toxicity problems encountered during the clinical development of camptothecin [5–8], the analogue I was synthesized to obtain a more water-soluble and less toxic compound [9]. This camptothecin analogue presents interesting preclinical activity *in vitro*

TABLE III

BETWEEN-DAY PRECISION AND ACCURACY OF THE HPLC DETERMINATION OF CPT-11 (I) AND SN-38 (II) IN HUMAN PLASMA

Number of observations = 10; results are expressed as mean ± S.E.M.

Concentration (ng/ml)	I		II	
	R.S.D. (%)	Accuracy (%)	R.S.D.(%)	Accuracy (%)
5	12.7 ± 5.7	113.1 ± 22.9	14.3 ± 4.8	73.2 ± 4.7
25	4.4 ± 0.5	101.7 ± 4.2	7.2 ± 1.2	95.8 ± 2.3
50	9.8 ± 4.8	117.7 ± 13.3	12.2 ± 1.9	107.6 ± 5.2
1000	5.4 ± 1.7	103.3 ± 10.9	6.9 ± 3.8	101.4 ± 8.2
10000	7.1 ± 0.8	96.1 ± 4.5	8.0 ± 1.1	93.7 ± 2.9

and *in vivo* in various experimental systems, and is also active in pleiotropic drug-resistant tumours [9–11]. The mechanism of action of this analogue is presumed to be mediated through topoisomerase I inhibition [12].

To study the human pharmacokinetics of both I and II, as part of a phase I evaluation of this compound, we developed a sensitive HPLC assay, allowing the simultaneous determination of both compounds. The determination of the pharmacokinetics of II is of prime importance, as it is formed *in vivo* and it is about 100–400 times more potent than the parent drug [13,15].

Although an HPLC method that allowed the determination of both compounds separately has been reported [13], a simultaneous method is preferable because it saves time and materials, and also increases the precision of the measurements on the same sample by minimizing handling. Our simultaneous assay also minimizes the total blood volume needed for pharmacokinetic studies because it requires only about 100 μl of plasma per time point. In terms of detection limits, the simultaneous assay allows 1 ng/ml for both compounds, whereas separate methods allow the detection of 1 ng/ml for I and 5 ng/ml of II [13].

This is the first account of the relatively long terminal half-life of about 20 h for drug I. This long disposition half-life could be of therapeutic advantage for this S-phase specific drug, allowing prolonged exposure times for tumour cells *in vivo*. This prolonged disposition half-life is also probably responsible for the maintenance of cytotoxic concentrations of the active metabolite II *in vivo*. Interestingly, the terminal half-life of I seems longer than that of SKF-104864, another closely related camptothecin analogue currently under clinical development, which was reported to have a terminal half-life of about 3 h [16].

This assay has been implemented to determine the clinical pharmacokinetics of I and II during ongoing clinical phase I trials of this camptothecin analogue [17]. To date, the main toxicities observed with I were gastro-intestinal and haematological [17]. This novel camptothecin analogue has already shown promising clinical anticancer activity [18,19].

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