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High-performance liquid chromatographic determination of irinotecan (CPT-11) and its active metabolite (SN-38) in human plasma

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Abstract

A simplified method for the simultaneous determination of irinotecan (CPT-11, I) and its active metabolite (SN-38, II) in human plasma by high-performance liquid chromatography (HPLC) with fluorescence detection has been developed. Following the addition of the internal standard (I.S.) camptothecin, the drugs were extracted from plasma using methanol. The average extraction efficiencies were 87% for I, 90% for II and 90% for the I.S. Chromatography was performed using a TSK gel ODS-80Ts column, monitored at 556 nm (excitation wavelength, 380 nm) and the mobile phase was acetonitrile–50 m*M* disodium hydrogen phosphate (28:72) containing 5 m*M* heptanesulphonate (pH 3.0). The linear quantitation ranges for I and II were 30–2000 and 1–30 ng/ml, respectively.

1. Introduction

Irinotecan (7-ethyl-10-[4-(l-piperidino)-1-piperidino] carbonyloxycamptothecin, CPT-11, I, see Fig. 1) is one of a series of semisynthetic camptothecin (CPT, see Fig. 1) derivatives produced in an attempt to reduce the toxicity and improve the therapeutic efficacy of the parent compound by increasing its water solubility without opening the lactone ring [1].

In a Japanese phase I trial describing clinical pharmacological data [2–6], the researchers measured the plasma concentrations of I and its active metabolite (7-ethyl-10-hydroxycamptothecin, SN-38, II, see Fig. 1) using the high-performance liquid chromatographic (HPLC) method of Kaneda et al. [7]. However, in view of the future situation of the usage of I in clinical practice with plasma drug level monitoring, it would be extremely useful to be able to measure the levels of I and II simultaneously. Therefore, we started to develop new assay methods that

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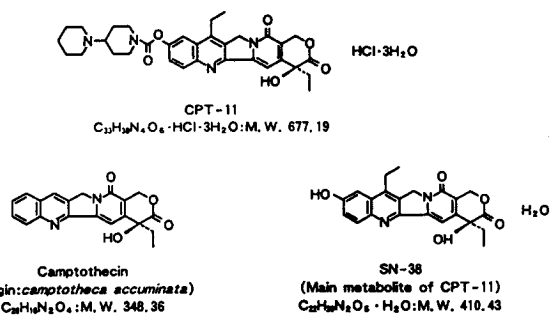

Fig. 1. Chemical structures of I, its active metabolite II and camptothecin (CPT, I.S.).

would enable us to do this before we started a phase II trial of cisplatin and I against small-cell lung cancer in 1992 [8]. During the preparation of this manuscript, two HPLC methods describing simultaneous determination of I and II were published, but these reports included no precise details of their precision and accuracy, especially for the determination of II, in the concentration ranges achieved with clinical usage [9,10].

The purpose of this study was to develop a simple and reliable HPLC method sensitive enough for pharmacokinetic studies and plasma level monitoring of I and its active metabolite (II) in clinical practice.

2. Experimental

2.1. Reagents and materials

The water, acetonitrile and methanol used were of HPLC grade and disodium hydroxygen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and orthophosphoric acid (H_3PO_4) were of analytical-reagent grade, all of which were purchased from Katayama Kagaku Kogyo (Osaka, Japan). 1-Heptanesulphonic acid sodium salt 1-hydrate (heptanesulphonate) was of HPLC grade and purchased from Eastman Fine Chemicals (Rochester, NY, USA). Yakult Honsha provided I and II and the internal standard (I.S.; CPT) was purchased from Sigma Chemical (St. Louis, MO, USA). Human control plasma samples were obtained from healthy male volunteers.

2.2. Apparatus and chromatographic conditions

Chromatographic determinations of I and II were performed using a Jasco Gulliver HPLC system (Nihon Bunko, Tokyo, Japan) that comprised the following: a PU 980 pump coupled to an automatic injector, 851-AS, with a 100-μl fixed loop and a 821-FP spectrofluorometer. Data output was monitored using a Jasco 807-1T integrator. The analytical column used was TSK gel ODS-80Ts (15.0 cm × 4.6 mm I.D.; 5-μm particle size) with a line-filter 014766E and a guard column of TSK guardgel ODS-120T (1.5 cm × 3.2 mm I.D.), all of which were covered by a column heater equipped with a thermomonitor (Yamato Kagaku, Tokyo, Japan). All the columns and the filter were purchased from Toso (Tokyo, Japan). The column flow-rate was 1.0 ml/min, the precolumn and column temperatures were maintained at 30°C and the usual analytical column pressure was 80–90 kg/m^2. The eluted peaks were monitored at 556 nm (the emission wavelength was 380 nm) and both concentrations I and II were calculated from the ratios of their peak areas to that of the I.S.

2.3. Mobile phase

The mobile phase was acetonitrile–50 m*M* disodium hydrogen phosphate (28:72, v/v) containing 5 m*M* heptanesulphonate, adjusted to pH 3.0 with orthophosphoric acid. The mobile phase was prepared by dissolving 17.9 g of disodium hydrogen phosphate 12-water and 1.1 g heptanesulphonate in 1000 ml water, 388 ml of acetonitrile was added and finally, the pH was adjusted.

2.4. Standard solutions

An amount of 10 mg of I was weighed accurately, transferred to a grade-A volumetric flask, dissolved in 10 ml water and then 2 ml water was added to 8 ml of this solution. The resulting solution was diluted to 80 μg/ml with 0.01 *M*

HCl and methanol was added to produce a final solution (stock 1) of 8 μg/ml of I. An amount of 10 mg of II was also accurately weighed, transferred to a grade-A volumetric flask, dissolved in 10 ml 0.1 *M* NaOH to produce a 1 mg/ml solution, which was diluted with water to 100 μg/ml. An aliquot of 8 ml methanol was added to 1 ml of this solution, followed by 1 ml 1 *M* HCl to produce a 10 μg/ml solution and, finally, 8.8 ml methanol was added to 1.2 ml of this solution to produce a final concentration of II of 1.2 μg/ml (stock 2). The I.S. solution was prepared by weighing 10 mg CPT accurately, transferring it to a grade-A volumetric flask and dissolving it in 10 ml 0.1 *M* NaOH. This solution was transferred to another volumetric flask and 90 ml water was added to produce a CPT concentration of 100 μg/ml. An amount of 1 ml 1 *M* HCl was added to 1 ml of this solution, followed by 8 ml methanol to produce a 10 μg/ml solution and finally, 8.75 ml methanol was added to 1.25 ml of this solution to produce a final concentration of 1.25 μg/ml (stock 3). All three stock standard solutions were stable for at least one year when stored at −80°C.

Working standard solutions were prepared by diluting these stock solutions with methanol and plasma standards were prepared by diluting appropriate aliquots of working standard solution with control human plasma.

2.5. Extraction and assay procedures

The sample for analysis was prepared as follows. An aliquot of 200 μl of sample plasma was placed in a 1.5-ml disposable conical polypropylene tube (Treff, Degerscheim/Schwelz, Switzerland), followed by 50 μl I.S. solution (CPT, 1.25 μg/ml) and 750 μl methanol. The tube was vortex-mixed for 10 s and centrifuged for 5 min at 10 000 *g* in a refrigerated microcentrifuge at 20°C. The upper aqueous layer was transferred to a new tube and evaporated under a vacuum using a CC-101 centrifugal concentrator (Tomy-Seiko, Tokyo, Japan). The dried extract was reconstituted with 400 μl mobile-phase solution adjusted to pH 2.0, vortexed for 10 s and then centrifuged for 5 min at 10 000 *g* in a refrigerated microcentrifuge at 20°C. Finally, the 100 μl resulting supernatant solution was injected into the column.

2.6. Recovery

The absolute recoveries of I, II and I.S. (CPT) were calculated by comparing the peak area obtained after injection of the theoretical amount of each compound with that obtained after injection of the extracts from the plasma samples.

2.7. Calibration

Calibration was carried out using the internal standard method. A calibration graph for each analyte was constructed by linear regression analysis of the analyte peak-area to I.S. peak-area ratios obtained using the least-squares method.

2.8. Evaluation of precision, accuracy and lower limit of quantitation

The overall assay performance was evaluated using all the individual values determined at each concentration point. The individual calculated concentrations were used to determine the relative standard deviation (R.S.D.) of the mean, relative mean error (R.M.E.) and total error (T.E.) [11] using the following equations: $\text{R.S.D.} = (\text{S.D.}/\text{mean}) \cdot 100$; $\text{R.M.E.} = \{(\text{mean} - \text{TCONC})/\text{TCONC}\} \cdot 100$; $\text{T.E.} = \{2\text{S.D.} + (\text{mean} - \text{TCONC})/\text{TCONC}\} \cdot 100$, in which S.D. is the standard deviation of the mean and TCONC the theoretical concentration of the analyte.

The R.S.D. and R.M.E. are indicators of precision and accuracy, respectively. The lower limit of quantitation (LOQ) for each analyte was set as the lowest standard concentration that had an R.S.D. < 20%, R.M.E. < 15% and T.E. < 50%. The upper LOQ was defined as either the highest concentration that met these criteria or the highest concentration point analyzed. These definitions of the LOQ values are in line with recently published guidelines [12].

2.9. Clinical samples

This assay was applied to the measurement of concentrations I and II in plasma following intravenous administration of I over 90 min in our phase II clinical trial [8]; some of these data are presented in this report. Blood samples were drawn 0, 0.5, 1, 1.5, 2, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 11.5 and 25.5 h after drug administration, heparinized and centrifuged at 10°C immediately after collection using a low-speed refrigerated centrifuge RL-130 (Tomy-Seikyo, Tokyo, Japan). The resulting plasma portions were stored at −80°C until subjected to analysis. Written informed consent was obtained from all the patients prior to entry into the study.

2.10. Statistical analysis

The statistical analysis was performed using computer program StatView II (Avacus Concepts, Berkeley, CA, USA) on a Macintosh PowerBook 170 microcomputer.

3. Results

3.1. Chromatography

Fig. 2 shows representative chromatograms obtained in our study. Assays of drug-free plasma showed there were no interfering endogenous peaks, neither was there any interference from other medications taken by these subjects. The retention times of I, II and CPT were approximately 5.4, 7.3 and 8.8 min, respectively, and the overall chromatographic run time was 11.5 min.

3.2. Extraction recovery

The I, II and I.S. (CPT) were extracted from plasma by a methanol extraction; their re-

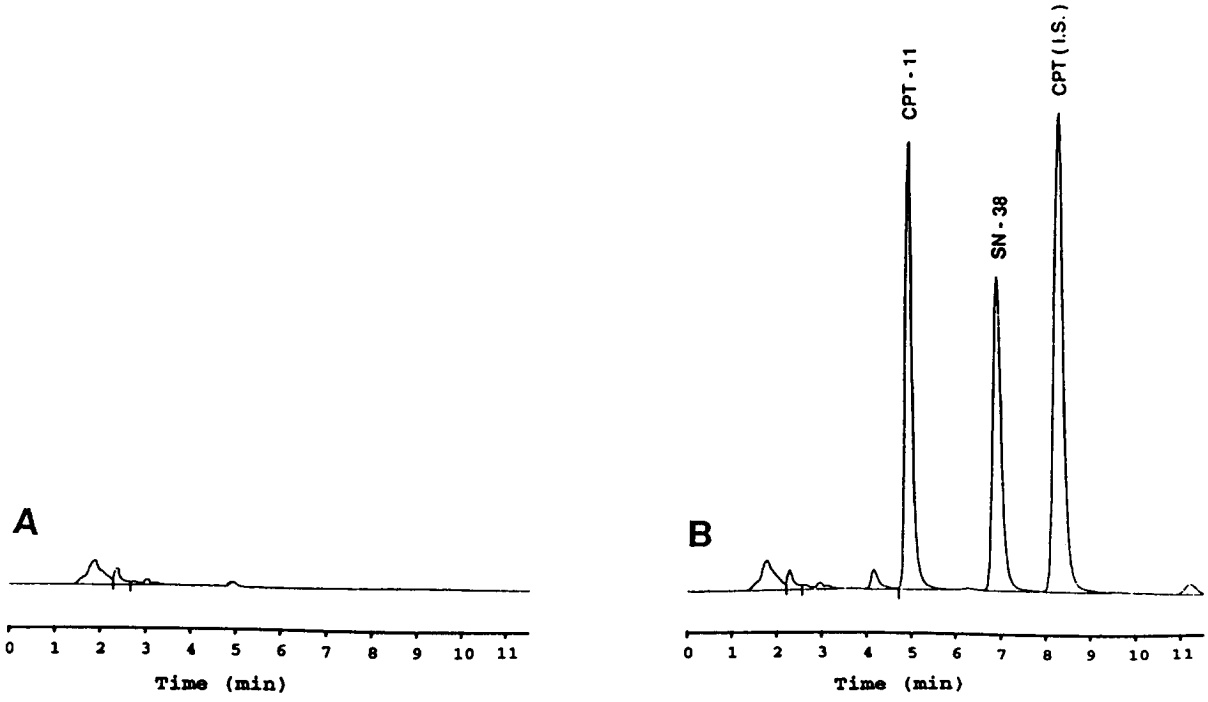

Fig. 2. Chromatograms of extracts obtained from (A) 200 μl drug-free human plasma and (B) 200 μl plasma spiked with I (300 ng/ml), II (20 ng/ml) and the internal standard, CPT (312.5 ng/ml).

Table 1
Extraction recoveries of I and II

I		II	
Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)
20	93.1 ± 1.3	1	93.9 ± 4.1
30	87.3 ± 11.3	2	88.8 ± 2.4
100	87.6 ± 5.0	5	87.3 ± 2.7
300	86.4 ± 4.8	10	89.6 ± 0.8
1000	86.8 ± 2.5	20	88.0 ± 1.1
2000	87.9 ± 2.0	30	88.3 ± 1.4

All values are means ± S.D. of four independent experiments.

coveries were approximately 90%. Table 1 shows the absolute recoveries of I and II, calculated by direct comparison of the peak areas of the plasma standards with those of the corresponding standards in solvent. The mean ± S.D. absolute recovery of the I.S. (312.5 ng/ml plasma) was 89.8 ± 2.9%.

3.3. Linearity

The calibration graphs for I and II were linear from 20 to 2000 and from 1 to 30 ng/ml, respectively. The means ± S.D. of their calibration graphs yielded the following equations: $y = (2.0741 \pm 0.0734)x + (0.0068 \pm 0.0068)$ for I and $y = (0.0312 \pm 0.0017)x + (-0.0024 \pm 0.0021)$ for II, in which y is the ratio of the peak area of I or II to that of the I.S. and x is the concentration (ng/ml) of I or II.

3.4. Assay validation

Tables 2 and 3 show the within-day and between-day, respectively, precision (R.S.D.), accuracy (R.M.E.) and T.E. for the limits of quantitation (LOQ) for each analyte. The precision and accuracy of this method were acceptable. The LOQ for I was 30 ng/ml and that for II was 1 ng/ml, which are in accordance which the recently published guidelines [12].

Table 2
Within-day reproducibility of the assay for I and II

I				II			
Concentration (ng/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)	Concentration (ng/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)
20	4.8	11.3	21.9	1	2.7	10.0	16.0
30	5.4	7.9	19.5	2	7.0	0.5	14.5
100	1.7	3.5	7.1	3	0.7	0.3	1.7
300	5.6	5.2	16.9	5	1.8	−2.0	1.6
1000	4.7	−2.3	6.9	10	1.9	−2.2	1.6
2000	0.9	−1.2	0.4	20	4.5	−3.0	5.8
				30	1.6	−2.5	0.7

All values are means ± S.D. of six independent experiments. R.S.D. = relative standard deviation; R.M.E. = relative mean error; T.E. = total error.

Table 3
Between-day reproducibility of the assay for I and II

I				II			
Concentration (ng/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)	Concentration (ng/ml)	R.S.D. (%)	R.M.E. (%)	T.E. (%)
20	24.0	−6.2	39.0	1	9.8	12.0	34.0
30	11.1	−1.8	20.1	2	10.5	4.5	26.5
100	5.5	−2.0	8.8	3	2.3	1.7	6.3
300	1.6	7.0	10.4	5	1.4	−2.2	0.6
1000	1.8	−0.6	2.9	10	2.6	−2.3	2.7
2000	2.1	−1.2	3.0	20	4.8	−3.4	5.8
				30	1.6	−2.1	1.0

All values are means ± S.D. of nine independent experiments. R.S.D. = relative standard deviation; R.M.E. = relative mean error; T.E. = total error.

3.5. Clinical applicability

This method was applied to a pharmacokinetic study of I and II in patients with small-cell lung cancer. Fig. 3A shows the plasma concentration versus time profiles of I and II after administration of I (60 mg/m^2) to a 62-year old Japanese male patient. Fig. 3B shows this profile after the same dose of I administered to a 68-year old Japanese female patient, who experienced Eastern Cooperative Oncology Group (ECOG) grade 4 neutropenia, thrombocytopenia and grade 3 diarrhoea, both of which are thought to be dose-limiting toxicities of I. It should be noted that there was a second peak in the concentration–time curve of compound II of this patient between 8 to 12 h after starting administration of I.

4. Discussion

This report describes a simple, accurate, precise and specific HPLC method for the simultaneous measurement of the concentration of I and II in human plasma.

Although the respective optimum excitation and emission wavelengths are 370 and 430 nm for I and 380 and 556 nm for II, we selected the latter two wavelengths to obtain the maximum sensitivity for II. As the range of the concentrations of II after the administration of 60–90 mg/m^2 of I in the previous Japanese combination phase I study was far lower (1–20 ng/ml) than that of I (100–2000 ng/ml), it was necessary to devise a method more sensitive to II to monitor the two compounds simultaneously. Furthermore, we have data indicating that the concentrations of standard I solutions (20, 300, 2000 ng/ml) measured at 430 nm (excitation wavelength, 370 nm) and 556 nm (excitation wavelength, 380 nm) did not differ.

Barilero et al. [9] have published a similar HPLC method for the measurement of I and II. Although these authors described the analytical characteristics in detail and their assay was used successfully for therapeutic monitoring purposes, their method included several features that we wished to modify. These included a mobile phase pH of 4.0, which would make the lactone ring remain open during the HPLC run, and a lack of information about precision and accuracy in the range of the concentration of II of 5–25 ng/ml, which we usually obtained in clinical practice after administration of 60–80 mg/m^2 of I.

A similar method was also reported recently by Rothenberg et al. [10], but their description of the method was included only in the "Patients and methods" section of their phase I clinical trial report and lacks precise information. Therefore, in view of the precision, accuracy, linearity and lower limits of quantitation obtained in our

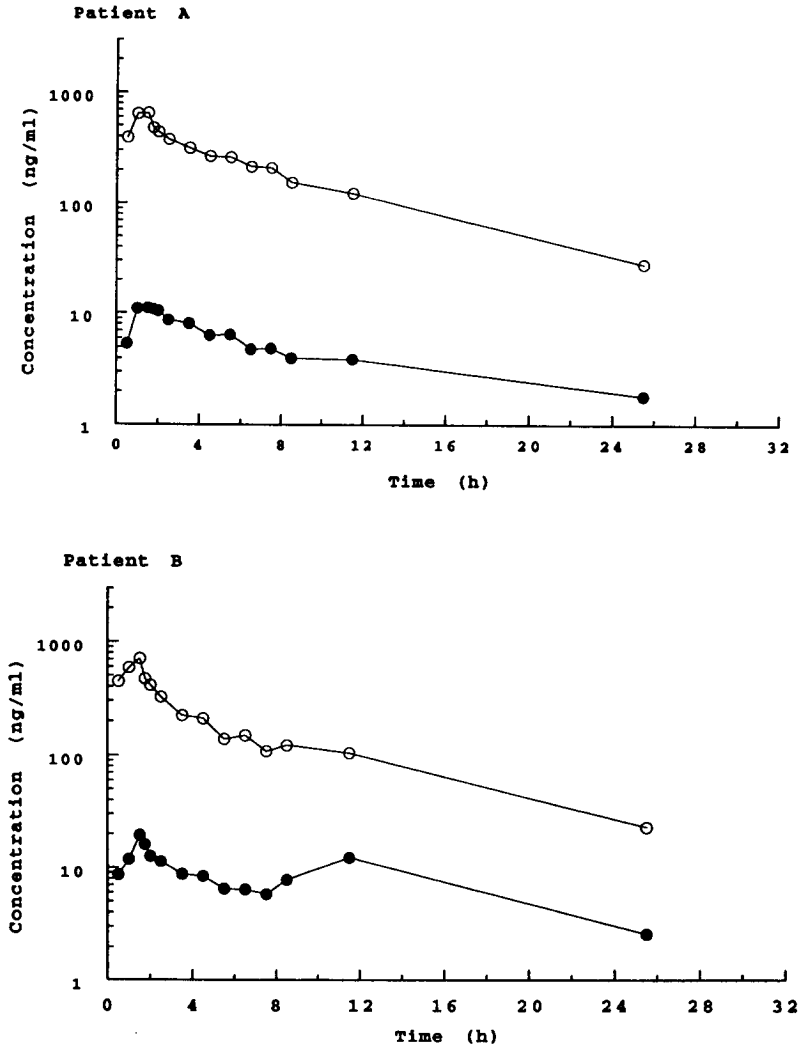

Fig. 3. Plasma concentration versus time profiles of I (○) and II (●) after infusion of 60 mg/m^2 of I over 90 min to two patients with small-cell lung cancer. Patient A was a 62-year old male, who received I, according to the above schedule, from time 0 (h). He experienced neither severe hematological nor non-hematological toxicity. Patient B was a 68-year old female, who was treated identically. She experienced ECOG grade 4 leukocytopenia, grade 4 thrombocytopenia and grade 3 diarrhoea.

study, we think that our method is valuable for patients who receive a relatively low dose (less than 100 mg/m^2) of I weekly.

In view of the fact that the areas under the concentration–time curves (AUC) for I and II correlate with hematological toxicity and the severity of diarrhoea observed in the previous phase I studies, it will be extremely important to monitor the plasma levels of both drugs by our simple HPLC method to prevent/predict these toxicities in clinical practice when I is approved for routine usage. Furthermore, we should pay attention to the existence of the second peak in the concentration–time curve of II, which indicates entero-hepatic circulation of this drug. We observed such a second peak in the curves of about 30% of the patients who received I in our phase II study. The major elimination pathway of II has been shown to be glucuronic acid conjugation and part of the glucuronide excreted

in bile is supposed to be hydrolysed by β-glucuronidase in the gut flora [7,13]. The second peak in the concentration–time curve of II is the first clear demonstration of entero-hepatic circulation of this drug in man.

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