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High-performance liquid chromatographic method for the simultaneous determination of the camptothecin derivative irinotecan hydrochloride, CPT-11, and its metabolites SN-38 and SN-38 glucuronide in rat plasma with a fully automated on-line solid-phase extraction system, PROSPEKT

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Abstract

We established a high-performance liquid chromatography (HPLC) method for the simultaneous determination of the camptothecin (CPT) derivative, irinotecan hydrochloride (CPT-11) and its metabolites, 7-ethyl-10-hydroxycamptothecin (SN-38) and SN-38 glucuronide (SN-38G) in rat plasma with a fully automated on-line solid-phase extraction system, PROSPEKT. Plasma samples were pretreated with 0.146 *M* H_3PO_4 to inactivate carboxylesterase and β-glucuronidase in rat plasma, and added with the internal standard solution (0.146 *M* H_3PO_4 containing 1 μg/ml CPT) and then analyzed. The method was validated for CPT-11 (5 to 25 000 ng/ml), SN-38 (5 to 2500 ng/ml) and SN-38G (2.5 to 500 ng/ml). This method enabled the determination of many samples within a relatively short time with easy sample preparation. It also had four advantages compared with conventional determination methods, i.e. automation of a complicated sample preparation, time-saving by the simultaneous determination of three compounds, the direct determination of SN-38G, and the small amount of plasma required for the determination.

Keywords: Camptothecin; Irinocetan hydrochloride; 7-Ethyl-10-hydroxycamptothecin

1. Introduction

Camptothecin (CPT), a plant alkaloid extract from *Camptotheca acuminata,* has strong antitumor activity due to its inhibition of the nuclear enzyme DNA topoisomerase I [1–4]. A water-soluble derivative of camptothecin, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, i.e. irinotecan hydrochloride (CPT-11, Fig. 1) was semi-synthesized to improve the antitumor activity as well as to decrease the toxicity of CPT [5,6]. CPT-11 has shown clinical activity against colorectal, lung, gastric, cervical, and ovarian cancers, malignant lymphoma, and other malignancies [7–10].

In the pharmacokinetic studies of CPT-11, several methods have been described for the measurement of CPT-11 and its metabolite, 7-ethyl-10-hydroxycamptothecin, SN-38, with the advanced automated sample processor (AASP) method and the extraction methods using a solid-phase column or methanol for

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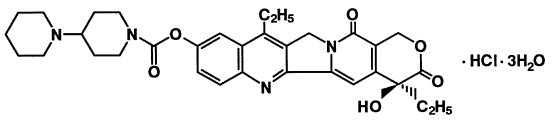
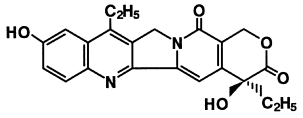
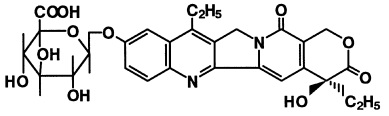


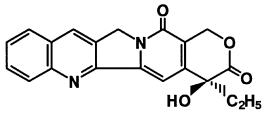
Irinotecan hydrochloride (CPT-11)



7-Ethyl-10-hydroxycamptothecin (SN-38)



SN-38 glucuronide (SN-38G)



Camptothecin (CPT)

Fig. 1. Structures of irinotecan hydrochloride (CPT-11), 7-ethyl-10-hydroxycamptothecin (SN-38), SN-38 glucuronide (SN-38G) and camptothecin (CPT).

sample preparation [11–13]. The AASP method is rather time-consuming, because separate determinations are necessary for CPT-11 and SN-38, and the other two reported extraction methods might not maintain adequate precision at low concentrations, because the methods require many manual operations. In addition, the determination of the conjugate of SN-38, SN-38 glucuronide (SN-38G), is both time-consuming and problematic regarding precision because it has been determined as SN-38 deconjugated by β-glucuronidase. Therefore, a method for the rapid and simple determination of CPT-11, SN-38 and SN-38G with high precision has been sought.

Several methods for the measurement of CPT-11 and SN-38 in plasma that recognize their lactone and carboxylate forms have recently been described [14–16]. However, the monitoring of total CPT-11 and total SN-38 has essentially the same clinical significance as the monitoring of lactone CPT-11 and lactone SN-38, because the pharmacokinetics of total CPT-11 and total SN-38 are significantly correlated with those of lactone CPT-11 and lactone SN-38, respectively [16,17]. Moreover, with these determination methods, it is impossible to determine the CPT-11 and SN-38 of many samples within a relatively short time, because the plasma has to be immediately treated after sampling, and treated samples have low stability. Therefore, conventional methods such as the AASP method and the extraction methods using a solid-phase column or methanol, which determine only the lactone forms of CPT-11 and SN-38 after the acidification of samples, are more appropriate to determine, for instance, samples in which the ratios of lactone forms to the total of CPT-11 and SN-38 are already known, or samples stored in freezer and pooled urine or bile samples in which the ratios of lactone forms to the total of CPT-11 and SN-38 might have changed.

This paper describes a rapid and simple high-performance liquid chromatography (HPLC) method for the simultaneous determination of CPT-11, SN-38 and SN-38G in rat plasma with a fully automated on-line solid-phase extraction system, PROSPEKT [18,19].

2. Experimental

2.1. Materials and reagents

CPT-11 (Lot 115002), SN-38 (Lot 30091R) and SN-38G were provided by Yakult Honsha Co. (Tokyo, Japan). Camptothecin (CPT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium 1-decanesulfonate was purchased from Tokyo Kasei Kogyo (Tokyo). The water used was of Milli-Q grade, and all other chemicals used were of analytical or HPLC grade and were obtained from commercial sources. Plasma was obtained by cardiac puncture from male Sprague-Dawley (SD) rats purchased from Japan SLC (Hamamatsu, Japan) under light ether anesthesia.

2.2. Apparatus

The HPLC system was composed of a Waters 616 pump and a 470 scanning fluorescence detector (Waters, Milford, MA, USA). The detector was set at

373 nm, 428 nm, and ×100 (excitation and emission wavelength, and gain, respectively) from 0 to 2.7 min (for the determination of SN-38G), at 380 nm, 540 nm, and ×100 from 2.7 to 3.8 min (for that of SN-38), and at 373 nm, 428 nm, and ×10 from 3.8 to 8.5 min (for that of CPT-11 and the internal standard (I.S.) (CPT)). The HPLC column was a Waters Symmetry column C18 (150 mm×4.6 mm I.D., 5 μm). The mobile phase flow-rate was 1.5 ml/min at 50°C. The mobile phase was 0.05 *M* KH_2PO_4–acetonitrile (70:30, v/v) containing 4 m*M* sodium 1-decanesulfonate (pH 3.5 with H_3PO_4). The automated solid-phase extraction system (PROSPEKT; Spark Holland, Emmen, The Netherlands) was on-line linked to the HPLC system. Fig. 2 summarizes the sample preparation procedure using the PROSPEKT system. A cartridge-C18 analytichem (Spark Holland) was used for the solid-phase extraction. One hundred microlitres of the prepared sample containing CPT-11, SN-38, SN-38G and I.S. was applied to the cartridge which was activated previously with methanol, water, and 0.01 *M* H_3PO_4 in turn. The cartridge was washed with 0.01 *M* H_3PO_4 to remove the plasma and contaminants. The analytes were then directly eluted from the cartridge to the analytical column with the mobile phase. The chromatogram was analyzed with the software program, Millenium 2010J Chromatography Manager (Waters).

2.3. Sample preparation

CPT-11, SN-38 and SN-38G were dissolved in methanol, dimethylsulfoxide and 0.146 *M* H_3PO_4 at the concentrations of 1, 1 and 0.1 mg/ml, respectively. These solutions were further diluted with methanol to give the concentrations of 5 to 50 000, 5 to 5000 and 1 to 1000 ng/ml, respectively. Twenty-five microlitres of these diluted solutions was then added to the mixture consisting of 50 μl of rat plasma, 0.2 ml of 0.146 *M* H_3PO_4 and 0.25 ml of I.S. solution (0.146 *M* H_3PO_4 containing 1 μg/ml CPT). The final concentrations of CPT-11, SN-38 and SN-38G in the prepared standard samples were 2.5 to 25 000, 2.5 to 2500 and 0.5 to 500 ng/ml in plasma, respectively. The standard samples were stored at 4°C in polypropylene tubes.

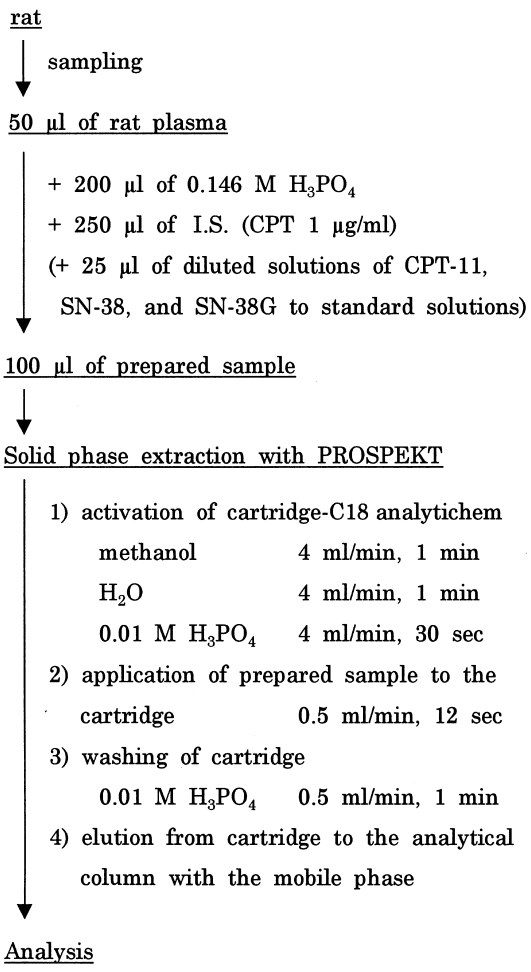


Fig. 2. Sample preparation procedure for rat plasma using the fully automated on-line solid-phase extraction system, PROSPEKT.

2.4. Calibration of the standard curves

The standard curve was established by plotting the ratio of the peak area to that of CPT against the concentration injected. A linear least squares regression analysis was conducted by weighing the reciprocal of the square of each concentration injected.

2.5. Method validation

Within-day and between-day validations were performed according to the methods of Shah et al. [20]. The accuracy and precision were determined

with five determinations per concentration. The mean values found should be within ±15% of the theoretical values, except for the lower limit of quantitation (LOQ), at which the deviation should be within ±20%.

The stability of CPT-11, SN-38 and SN-38G in the prepared samples was investigated by using the plasma obtained from a rat at 1 h after an intravenous (i.v.) administration of 40 mg/kg of CPT-11. The plasma was added to four volumes of 0.01 or 0.146 *M* H_3PO_4 and incubated at 4 or 37°C for 24 h. Part of the incubated sample was added with an equal volume of the I.S. solution and analyzed.

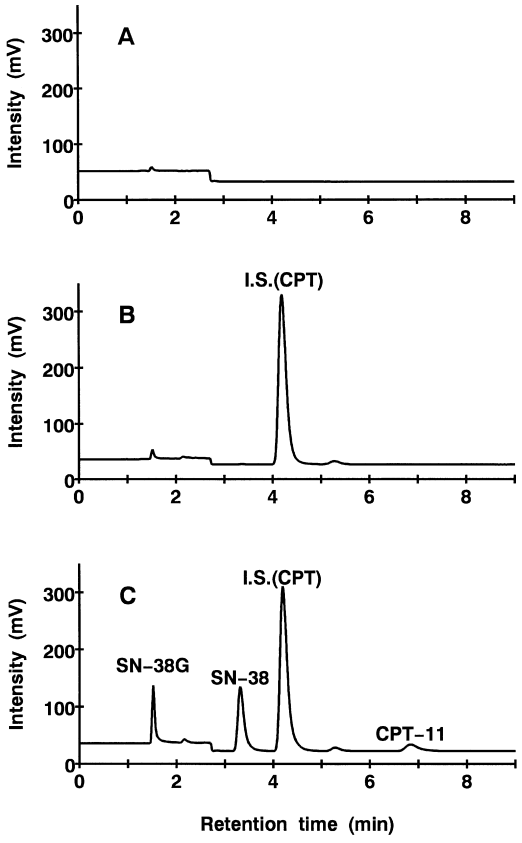


Fig. 3. Chromatograms of (A) blank plasma, (B) blank plasma+ I.S. (CPT), and (C) the plasma spiked with CPT-11 (500 ng/ml), SN-38 (500 ng/ml), SN-38G (50 ng/ml) and I.S. (CPT). The detector was set at 373 nm, 428 nm and ×100 (excitation and emission wavelength, and gain, respectively) from 0 to 2.7 min (for the determination of SN-38G), at 380 nm, 540 nm and ×100 from 2.7 to 3.8 min (for SN-38), and at 373 nm, 428 nm and ×10 from 3.8 to 8.5 min (for CPT-11 and I.S. (CPT)).

The freeze-thaw stability of CPT-11, SN-38 and SN-38G was investigated by using the standard samples. These samples of three or four different concentrations were stored at −20°C overnight and then thawed at room temperature. This operation was repeated three times and analyzed at each operation.

The recovery of CPT-11, SN-38 and SN-38G on the solid-phase extraction was investigated by comparing the peak area of the standard samples prepared with rat plasma and processed with the PROSPEKT system to those of the direct manually injected standard samples prepared with phosphate-buffered saline (PBS, pH 7.4) instead of rat plasma at three or four different concentrations.

2.6. Determination of the plasma samples after an i.v. administration of CPT-11 to rats

Male SD rats weighing 230 to 260 g were used. Cannulas (Intramedic PE-50; Clay Adams, Parsippany, NJ) were implanted under light ether anesthesia into the right femoral vein and the left femoral artery, through which CPT-11 was administered and blood was collected, respectively. The animals were kept in Bollman cages after the implantation, and 2, 10, 40 or 60 mg/kg of CPT-11 was administered, followed by flushing with physiological saline, after they awoke from anesthesia. Two hundred microlitres of blood was collected at 2, 5, 10, 30 and 60 min and every 3 h up to 24 h after the administration of CPT-11.

3. Results and discussion

3.1. Chromatograms

Camptothecin derivatives were sensitively analyzed by the detection of their fluorescence. The emission fluorescence wavelengths of the maximum responses (λ_{max}) for lactone CPT-11 and lactone SN-38 were 428 and 540 nm, respectively. In the present study, for the sensitive determination of CPT-11 and SN-38, we used a fluorescence detector that automatically changes the excitation and emission wavelengths suitable for the analytes during the analysis.

Fig. 3 shows chromatograms of blank plasma, the

plasma spiked with I.S. (CPT), and plasma spiked with CPT-11, SN-38, SN-38G and I.S. (CPT). CPT-11, SN-38, SN-38G and CPT were separated by this HPLC condition, and their retention times were 6.7, 3.2, 1.5 and 4.2 min, respectively. In the chromatogram of blank plasma, the peak was observed at 1.5 min, which might have interfered with the determination of SN-38G, but this peak was much smaller (0.2 ng equiv./ml) than that of the LOQ of SN-38G (2.5 ng/ml), and the peak thus little affected the determination of SN-38G (Table 3). No interfering peak was observed in the chromatogram of I.S. (CPT). Fig. 4 shows the chromatogram of the plasma sample obtained at 1 h after an i.v. administration of 40 mg/kg of CPT-11 to a rat. The two peaks observed at 2.2 and 5.2 min were contaminants in the I.S. solution. The peaks of CPT-11, SN-38, SN-38G and I.S. (CPT) were well separated from each other.

3.2. Optimization of sample preparation

Because the plasma of rats and mice has higher carboxylesterase activity than those of other animals [21], CPT-11 in rat plasma is rapidly converted to SN-38. The β-glucuronidase activity in rat plasma is reported to be elevated by an i.v. administration of a high dose of CPT-11 [22]; SN-38G in rat plasma might therefore be deconjugated to SN-38. Plasma samples were immediately acidified after sampling by the addition of four volumes of 0.146 *M* H_3PO_4, in order to inactivate these enzymes and to transform them to the lactone forms of CPT-11, SN-38 and SN-38G [14] and then analyzed. The pH of the acidified samples was about 1.5.

Fig. 5 shows the stability of CPT-11, SN-38 and SN-38G in the samples prepared with 0.01 *M* or 0.146 *M* H_3PO_4 and incubated at 4 or 37°C. The plasma used in this examination was sampled at 1 h

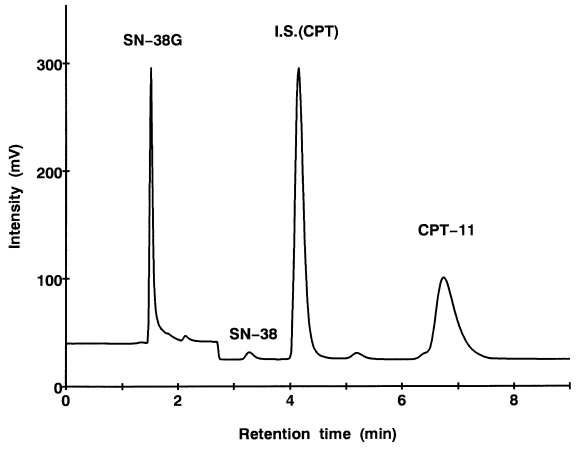


Fig. 4. Typical chromatogram of the plasma sample. The plasma sample was obtained at 1 h after an i.v. administration of 40 mg/kg of CPT-11 to a rat. The detector was set at 373 nm, 428 nm and ×100 (excitation and emission wavelength, and gain, respectively) from 0 to 2.7 min (for the determination of SN-38G), at 380 nm, 540 nm and ×100 from 2.7 to 3.8 min (for SN-38), and at 373 nm, 428 nm and ×10 from 3.8 to 8.5 min (for CPT-11 and I.S. (CPT)).

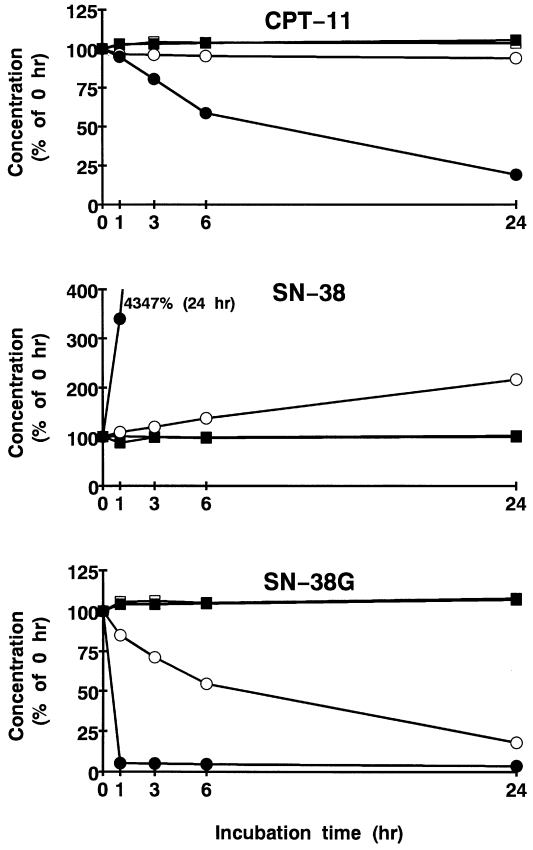


Fig. 5. Stability of CPT-11, SN-38 and SN-38G in the plasma sample at 1 h after an i.v. administration of 40 mg/kg of CPT-11 to a rat. Plasma samples were diluted with four volumes of 0.01 *M* or 0.146 *M* H_3P0_4, and incubated at 4 or 37°C for 24 h. Parts of the incubated samples were added to an equal volume of I.S. solution at 1, 3, 6 and 24 h after the start of incubation and analyzed. Data points are means of three samples. □; 0.146 *M* H_3P0_4 (4°C), ■; 0.146 *M* (37°C), ○; 0.01 *M* (4°C), ●; 0.01 *M* (37°C).

Table 1
Recovery of CPT-11, SN-38 and SN-38G in rat plasma samples by solid-phase extraction with the PROSPEKT system

	Concentration (ng/ml)	Peak area[a]		Recovery[b] (%)
		Manual injection	PROSPEKT	
CPT-11	5	6044±1091	7087±556	117.3
	50	33602±802	32649±360	97.2
	500	311033±850	309243±8311	99.4
	5000	3324637±1376	3190736±40184	96.0
SN-38	5	24459±127	26133±963	106.8
	50	156049±1847	151312±1173	97.0
	500	1483798±17900	1446014±16824	97.5
SN-38G	5	83961±4473	100413±6690	119.6
	50	621033±15419	616218±5812	99.2
	500	5511481±50616	5300649±17833	96.2

[a] Number of observations=three; results are expressed as mean±SD.
[b] Recovery (%)=(peak area of PROSPEKT)×100/(peak area of manual injection).

after an i.v. administration of 40 mg/kg of CPT-11. The initial concentrations of CPT-11, SN-38 and SN-38G were 6.2 μg/ml, 56.6 and 181.9 ng/ml, respectively.

In the plasma sample added with 0.01 *M* H_3P0_4 and incubated at 37°C for 24 h, the concentrations of CPT-11 and SN-38G were decreased to 19 and 4% of those at the start of incubation, respectively, and that of SN-38 was increased by forty-fold. In contrast, in the sample added with 0.146 *M* H_3PO_4, regardless of whether it was incubated at 4 or 37°C for 24 h, the concentrations of CPT-11, SN-38 and SN-38G were equal to those at the start of incubation. It was thus demonstrated that two enzymes (carboxylesterase and β-glucuronidase) in rat plasma were inactivated by this preparation method, and CPT-11, SN-38 and SN-38G in the prepared samples were stable at 37°C for 24 h.

Table 2
Within-day precision and accuracy of the HPLC determination of CPT-11, SN-38 and SN-38G in rat plasma[a]

	Theoretical concentration (ng/ml)	Mean measured concentration (ng/ml)	RSD[b] (%)	Accuracy (%)
CPT-11	5	5.0	14.2	100.8
	25	24.0	1.9	96.0
	250	253.8	0.0	101.5
	2500	2430.1	0.1	97.2
	25 000	25 144.1	0.2	100.6
SN-38	5	4.7	1.6	93.4
	25	24.0	0.1	95.8
	250	243.1	0.9	97.2
	2500	2538.8	0.8	101.6
SN-38G	2.5	2.4	3.4	95.2
	5	5.2	1.6	103.7
	50	51.1	0.8	102.1
	500	459.3	0.7	91.9

[a] Number of observations=five.
[b] RSD: relative standard deviation.

3.3. Recovery on the solid-phase extraction with the PROSPEKT system

The recovery of CPT-11, SN-38 and SN-38G of the prepared sample on the solid-phase extraction with the PROSPEKT system is summarized in Table 1. The peak areas of CPT-11, SN-38 and SN-38G in the plasma samples processed by the PROSPEKT system in comparison with those processed by manual injection were 117.3, 106.8 and 119.6% at the LOQ and within 96 to 100% at higher concentrations, respectively, suggesting that CPT-11, SN-38 and SN-38G in the prepared samples were almost completely recovered by this extraction method.

3.4. Standard curves

Good standard curves were obtained for CPT-11, SN-38 and SN-38G, ranging from 5 to 25 000, 5 to 2500 and 2.5 to 500 ng/ml, respectively, which were sufficient for the pharmacokinetic studies of CPT-11. The standard curve equations of CPT-11 were $Y=0.00335\ X+0.01155$ (peak area ratio $\leqq 0.036$), $r=0.999998$, and $Y=0.003775\ X-0.01130$ (peak area ratio >0.036), $r=0.999998$. Those of SN-38 and SN-38G were $Y=0.01635\ X+0.01330$, $r=1.000000$, and $Y=0.0645\ X+0.0560$, $r=0.999998$, respectively, where X and Y were the concentration (ng/ml) and the peak area ratio, respectively.

3.5. Validation

The within-day and between-day validation results of CPT-11, SN-38 and SN-38G are listed in Tables 2 and 3, respectively. For the within-day validation, the precision values (relative standard deviation; RSD) for CPT-11 was 14.2% at the LOQ and within 1.9% at higher concentrations, and the accuracy value (relative % error) was within 4.0% at each concentration. The precision values for SN-38 and SN-38G were within 1.6 and 3.4%, and the accuracy values were within 6.6 and 8.1% at each concentration range, respectively. For the between-day validation, the precision values of CPT-11, SN-38 and SN-38G were within 7.6, 2.4 and 5.0%, and the accuracy values were within 3.7, 3.3 and 10.5%, respectively.

3.6. Freeze–thaw stability

The freeze–thaw stability of CPT-11, SN-38 and SN-38G in the samples prepared with rat plasma is summarized in Table 4. The variation of CPT-11,

Table 3
Between-day precision and accuracy of the HPLC determination of CPT-11, SN-38 and SN-38G in rat plasma[a]

	Theoretical concentration (ng/ml)	Mean measured concentration (ng/ml)	RSD[b] (%)	Accuracy (%)
CPT-11	5	5.1	7.6±4.8	102.7±6.5
	25	24.1	3.6±2.0	96.3±2.6
	250	251.5	1.5±1.8	100.6±1.0
	2500	2410.8	0.3±0.2	96.4±0.6
	25 000	25 761.6	0.2±0.2	103.0±1.9
SN-38	5	5.1	2.4±1.7	103.0±8.9
	25	24.2	0.9±0.7	96.7±0.8
	250	242.6	1.0±0.1	97.1±0.4
	2500	2527.9	1.0±0.2	101.1±0.8
SN-38G	2.5	2.6	5.0±5.4	104.9±7.9
	5	5.4	2.7±1.9	108.3±4.1
	50	51.3	1.6±0.9	102.5±3.6
	500	447.7	1.3±1.6	89.5±5.9

[a] Number of observations=five; results are expressed as mean±SD.
[b] RSD=relative standard deviation.

Table 4
Freeze–thaw stability of CPT-11, SN-38 and SN-38G in rat plasma samples[a]

	Concentration (ng/ml)	Recovery from the initial (%)		
		Freeze cycle no.		
		1	2	3
CPT-11	5	95.6	94.2	101.7
	50	100.3	101.2	100.9
	500	101.5	98.6	94.8
	5000	98.4	98.6	98.4
SN-38	5	95.1	95.1	99.4
	50	99.6	101.6	101.3
	500	101.2	101.7	101.9
SN-38G	5	99.0	98.7	95.5
	50	100.4	98.7	97.3
	500	99.3	97.0	96.6

[a] Number of observations=three.

SN-38 and SN-38G in three freeze–thaw operations at each concentration was within 5.2, 4.9 and 4.5%, respectively, suggesting that these compounds were stable during the three freeze–thaw cycles.

3.7. Plasma concentration profiles of CPT-11, SN-38 and SN-38G in rats

Fig. 6 shows the plasma concentration-time curves of CPT-11, SN-38 and SN-38G after an i.v. administration of CPT-11 at doses of 2, 10, 40 and 60 mg/kg. By using this determination method, plasma concentrations of CPT-11, SN-38 and SN-38G after an i.v. administration of 2 mg/kg of CPT-11 were determined at 24, 6 and 24 h after administration, respectively. The smaller amount of plasma required for the determination enabled many sampling points. From these results, our new determination method was demonstrated to be suitable for the pharmacokinetic studies of CPT-11 and its two metabolites.

4. Conclusion

In this study, we established a method for the simultaneous determination of CPT-11, SN-38 and SN-38G which meets the criteria of Shah et al. [20] at the concentration ranges of 5 to 25 000, 5 to 2500 and 2.5 to 500 ng/ml, respectively. Compared with the conventional methods (Table 5), this method has several advantages, i.e. the rapid and simple sample preparation with a fully automated on-line solid-phase extraction system, PROSPEKT, time-saving by the simultaneous determination of three com-

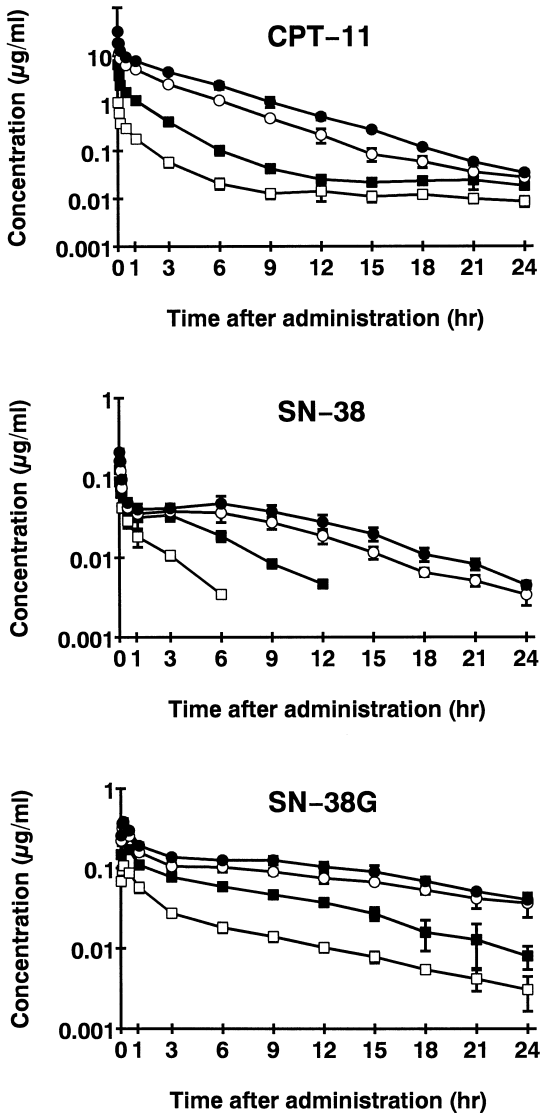


Fig. 6. Plasma concentration profiles of CPT-11, SN-38 and SN-38G after an i.v. administration of CPT-11 to rats. Male SD rats were i.v. administered CPT-11 at doses of 2 (□), 10 (■), 40 (○) and 60 mg/kg (●) through cannulas implanted in the right femoral vein. Data points are means±SD of four animals.

Table 5
Comparison of the new determination method for CPT-11, SN-38 and SN-38G with the conventional methods

Compound determined	Kaneda et al. [11]		Barilero et al. [12]		Tanaka et al. [13]		New method		
	CPT-11	SN-38	CPT-11	SN-38	CPT-11	SN-38	CPT-11	SN-38	SN-38G
Sample preparation and injection to the analytical column	Solid-phase extraction and direct elution to the column with the mobile phase automatically by AASP		Solid-phase extraction, evaporation of eluent, re-dissolution, and injection		Deproteinization with methanol, evaporation of supernatant, re-dissolution, and injection		Solid-phase extraction and direct elution to the column with the mobile phase automatically by PROSPEKT		
Determination[a]	Separately		Simultaneously		Separately		Simultaneously		
Analytical time	6.6 min[b]	13.8 min[b]	10 min		11 min	9 min	9 min		
Limit of quantitation	1 ng	5 ng	1 ng/ml[c]	1 ng/ml[c]	12.3 ng/ml	0.75 ng/ml	5 ng/ml	5ng/ml	2.5 ng/ml
Determination of SN-38G	As SN-38 after deconjugation		–		–		Directly		
Sample volume	300 μl (blood)		–		200 μl (plasma)		50 μl (plasma)		

[a] CPT-11 and SN-38 were determined simultaneously or separately.
[b] These values were the retention times of CPT-11 and SN-38 in the method of Kaneda et al. [11].
[c] The within-day accuracy of the determinations of CPT-11 and SN-38 did not meet the criteria of Shah et al. [20].

pounds, and the direct determination of SN-38G. The use of PROSPEKT enabled the processing of many samples within a relatively short time and the higher repeatability of determination regarding precision and accuracy compared to manual sample preparation, and the use of the automatic wavelength change fluorescence detector enabled the exact determinations of CPT-11 and SN-38 at low concentrations. The direct determination of SN-38G described in this report also gave more accurate value of SN-38G, even when the concentration of SN-38G was much lower than that of SN-38; at this concentration, SN-38G was often calculated as a negative value by the conventional methods. In addition, this method induces less stress in animal subjects compared to the conventional method because only a small amount of plasma (blood) was required for the determination.

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