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High-performance liquid chromatographic assay with fluorescence detection for the simultaneous measurement of carboxylate and lactone forms of irinotecan and three metabolites in human plasma

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Abstract

Irinotecan (CPT-11), a camptothecin analog, is metabolized to SN-38, an active topoisomerase I inhibitor, and inactive metabolites, including APC and SN-38 glucuronide (SN-38G). A high-performance liquid chromatographic assay method to simultaneously measure the lactone and carboxylate forms of CPT-11, SN-38, SN-38G, and APC in human plasma was developed. Chromatography was accomplished with a reversed-phase C_8 column and fluorescence detection. A gradient mobile phase system was used. The buffer for mobile phase A consisted of 0.75 *M* ammonium acetate, 5 m*M* tetrabutylammonium phosphate (pH 6.0), and acetonitrile (86:14, v/v). The buffer for mobile phase B was identical to mobile phase A with the exception of the concentration (50:50, v/v). Precipitation of plasma proteins was performed with cold methanol. The linear range of detection of the lactone and carboxylate forms of SN-38, SN-38G, and APC was 2–25 ng/ml, and 5–300 ng/ml for CPT-11. The limit of quantitation for the analytes ranged from 0.5 to 5 ng/ml. Analysis of patients' plasma samples obtained before and after CPT-11 administration showed that the assay is suitable for measuring lactone and carboxylate forms of CPT-11, SN-38, SN-38G, and APC in clinical studies.

Keywords: Irinotecan; SN-38

1. Introduction

The antitumor activity of 20(*S*)-camptothecin, an alkaloid isolated from the plant *Camptotheca acuminata*, has been recognized for more than 30 years. Although the drug showed promising antitumor activity in early phase I studies, its marked toxicity in phase II trials precluded its further development [1,2]. In recent years, irinotecan (CPT-11), a derivative of camptothecin, has proved to be among the most effective compounds tested for treating human cancer. CPT-11 is a prodrug in vivo, undergoing de-esterification by carboxylesterases to yield an active metabolite, SN-38. SN-38 interacts with the topoisomerase–DNA complex and prevents the religation of DNA single-strand breaks mediated by topoisomerase I. Glucuronidation of SN-38 by uridine diphosphate glucuronosyltransferase (UGT) yields SN-38 glucuronide (SN-38G) (Fig. 1). The relationship between SN-38 and SN-38G has been

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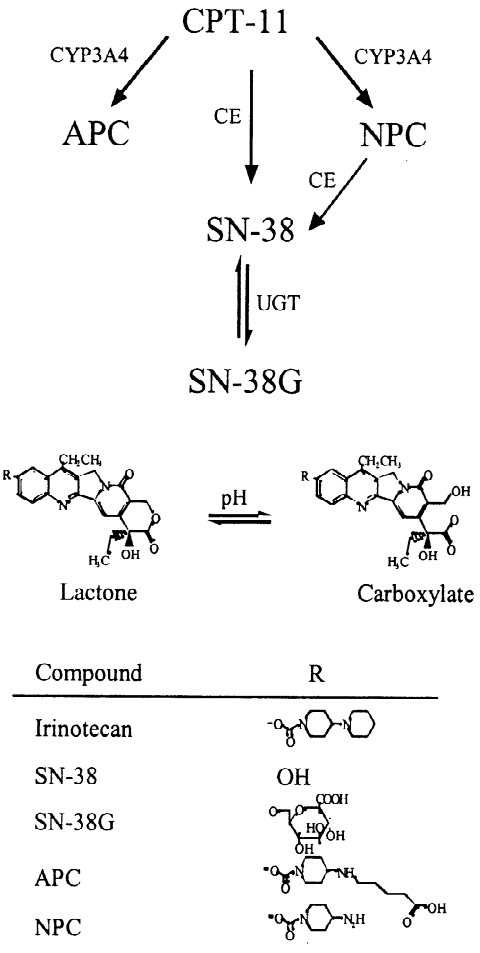

Fig. 1. Metabolic pathway and structures of CPT-11 and its major metabolites SN-38, SN-38G, APC, and NPC. CYP3A4, cytochrome P4503A4; CE, carboxylesterase; UGT, uridine diphosphate glucuronosyltransferase.

shown to be a significant predictor of toxicity in clinical studies [3]. Other metabolic pathways for CPT-11 include oxidation by cytochrome P450 3A4 (CYP3A4) [4]. Major oxidation products produced by this pathway are APC and NPC, both of which are weak inhibitors of topoisomerase I. The parent compound, SN-38, SN-38G, and APC accounted for 93% of the recovered dose in a radiochemical mass-balance study [5].

In vitro studies have shown that the closed lactone configuration of camptothecin analogs is the pharmacologically active form [6]. However, like the E-rings of other camptothecin analogs, the closed lactone rings of CPT-11 and its metabolites undergo pH-dependent reversible hydrolysis to open carboxylate forms.

The pharmacokinetics and pharmacodynamics of CPT-11 are complex because of the dynamic nature of the lactone-carboxylate conversion and because of the multiple metabolism pathways involving varied enzyme systems. Characterization of the opening of the lactone ring of CPT-11 and its metabolites requires methods that allow the quantitation of the lactone and carboxylate concentrations. Current high-performance liquid chromatography (HPLC) methods to measure CPT-11, SN-38, and SN-38G either require two injections [7], indirectly measure SN-38G concentrations after incubation with β-glucuronidase [3], or measure total (lactone+ carboxylate) concentrations but not the concentrations of the active lactone species [8–10]. The hydrolysis of lactone to its corresponding carboxylate can be stopped after a sample is obtained with immediate cooling of the blood, centrifugation and protein precipitation [11]. Using this protein precipitation method, Rivory and Robert first published a method to simultaneously measure the lactone and carboxylate forms of CPT-11 and SN-38 in patient plasma [12]. We have improved upon this method by developing a reversed-phase HPLC method to simultaneously measure the individual lactone and carboxylate forms of CPT-11, SN-38, SN-38G, and APC in a single run. In addition to evaluating the precision and accuracy of the assay, we demonstrated the application of the method using human plasma samples from a patient treated with CPT-11.

2. Experimental

2.1. Chemicals and reagents

CPT-11, APC, and SN-38 were supplied by Pharmacia (Kalamazoo, MI, USA). Analytical grade SN-38G was a gift from Yakult Honsha (Tokyo, Japan). All reagents and the organic solvents were of the highest analytical grade. Citric acid monohydrate, sodium borate, and glacial acetic acid were pur-

chased from Fisher Scientific (Fair Lawn, NJ, USA), tetrabutylammonium phosphate was from Waters (Milford, MA, USA), methanol and acetonitrile (UV grade for HPLC) were from Burdick and Jackson (Muskegon, MI, USA), ammonium acetate and hydrochloric acid were from Sigma (St. Louis, MO, USA), and 47-mm nylon-66 filters (pore size 0.20 μm) used for filtering the mobile phase were from Varian (Harbor City, CA, USA). Water was purified in a Milli-QUV Plus System (Millipore, Bedford, MA, USA) at 18.2 MΩ; this deionized water is referred to as ''water'' hereafter.

2.2. Instruments

The high-performance liquid chromatograph was an automated system that consisted of two Shimadzu LC-10AD VP gradient system pumps (Columbia, MD, USA), a Shimadzu SCL-10A VP system controller, a Shimadzu SIL-10AD auto-injector with a sample cooler kept at 4 °C, and a Shimadzu RF10AXL fluorescence detector. The data were acquired and analyzed by Shimadzu Class-VP (version 5.03) software.

2.3. Chromatographic conditions

Chromatographic separations were achieved by the use of a Symmetry C_8, 5-μm particle size, 3.9×150 mm I.D., analytical column with a Symmetry C_8, 5-μm, 3.9×20 mm sentry guard column (Waters, Milford, MA, USA), and a mobile phase gradient. Mobile phase A consisted of 86% of 0.75 *M* ammonium acetate, 5 m*M* tetrabutylammonium phosphate (pH 6.0), and 14% acetonitrile. Mobile phase B consisted of 50% of 0.75 *M* ammonium acetate, 5 m*M* tetrabutylammonium phosphate (pH 6.0), and 50% acetonitrile. The mobile phase was filtered through a Varian 47-mm nylon-66 filter (pore diameter 0.20 μm), and placed onto the pump to equilibrate. The flow rate of 1.25 ml/min remained constant, and the pressure remained less than 13.1 MPa throughout the run. The samples were eluted on a gradient with 100% mobile phase A for the first 15 min, after which time an 8-min linear gradient to 40% mobile phase B was initiated and continued at 40% until 28 min. Thereafter, the mobile phase was returned to 100% mobile phase A by 30 min and 10 min were allotted for baseline stabilization. The excitation wavelength remained stationary at 380 nm with a gain of 4. We allowed the emission wavelength to vary according to each compound's optimal wavelength, which was determined by spectrophotometry. At the start of each run, the emission wavelength was set at 460 nm; at 9.95 min the emission wavelength changed to 520 nm, until 13.4 min when it changed to 460 nm. At 17.3 min, the wavelength changed to 530 nm, where it remained until end of the run. Auto-injections were made every 40 min with an injection volume of 100 μl.

2.4. Stock solution, calibrators, and control samples

Stock solutions of CPT-11, APC, and SN-38G were prepared in 0.01 N HCl. The concentration of the CPT-11 and APC stock solutions was 1 mg/ml and that of the SN-38G stock solution was 0.1 mg/ml. The light sensitive stock solution of SN-38 was made by dissolving 0.1 mg of SN-38 powder in 1 ml of methanol. All stock solutions were stored at −70 °C.

We used the stock solutions to prepare working standard solutions. A separate working standard solution was made for each lactone and carboxylate calibrator and control sample for CPT-11 lactone (CPT-11 L), CPT-11 carboxylate (CPT-11 C), SN-38 lactone (SN-38 L), SN-38 carboxylate (SN-38 C), SN-38G lactone (SN-38G L), SN-38G carboxylate (SN-38G C), APC lactone (APC L), and APC carboxylate (APC C). Working standard solutions were made fresh before they were added to the diluent, and the concentrations of the solutions were 100 times that of the appropriate calibrator or control sample. The working standard solutions for CPT-11 L, APC L, and SN-38G L were prepared by diluting the appropriate stock solutions (1, 1 and 0.1 mg/ml, respectively) with 0.05 N citric acid. The working standard solution for SN-38 L was prepared by diluting the SN-38 stock solution (0.1 mg/ml) with methanol. The working standard solutions for CPT-11 C, APC C, SN-38G C, and SN-38 C were prepared by diluting the appropriate stock solutions

(1, 1, 0.1 and 0.1 mg/ml, respectively) with 0.04 N borax.

2.5. Sample preparation

Drug-free human plasma from a single donor (Mid-South Regional Blood Center, Memphis, TN, USA) was used as the vehicle for the calibrators and control samples. After all working standard solutions were prepared, we inserted 460 μl of human plasma into a 1.5-ml Eppendorf tube and placed the tubes on ice. A volume of 5 μl of each appropriate working standard solution was added to the plasma; the final volume of the plasma mixture was 500 μl. The order in which the working standard solutions were added to the plasma was as follows: SN-38 L, SN-38G L, SN-38 C, SN-38G C, APC L, APC C, CPT-11 L, and CPT-11 C. Each sample was vortex mixed after each working standard solution was added to the plasma. The final 500-μl sample was vortex mixed for 10 s in a polypropylene centrifuge tube. Two 200-μl volumes of this plasma mixture were added to duplicate tubes containing 800 μl of cold methanol; the mixtures were then centrifuged for 2 min at 7000 *g*. The supernatant was decanted into a polypropylene screw-top tube. For the calibrators SN-38 L, SN-38 C, SN-38G L, SN-38G C, APC L, and APC C, we used the following concentrations to determine the linear range of detection: 2, 5, 7.5, 15, and 25 ng/ml. To determine the linear range of detection for CPT-11 L and CPT-11 C, we used calibrator concentrations of 5, 25, 50, 100, and 300 ng/ml. Duplicates of each calibration standard were analyzed. The calibration curve underwent linear regression analysis in which the peak heights were plotted in relationship to the inverse squares of the concentrations of the calibrators. Concentrations of the quality control samples for SN38 L, SN-38 C, SN-38G L, SN-38G C, APC L, and APC C were 3 and 10 ng/ml; those for CPT-11 L and CPT-11 C were 15 and 75 ng/ml. The calibrators and quality control samples were stored at −70 °C until analysis.

Before a calibrator, a control, or a patient sample was injected, 45 μl of the methanol-extracted sample to be injected was added to 90 μl of mobile phase A buffer. This mixture was vortex mixed for 3 s and pipetted into an autosampler tube. A 100-μl aliquot of this mixture was injected onto the column.

2.6. Precision and accuracy

Intra-day and inter-day precision and accuracy of the assay were determined by analyzing plasma replicates containing known low and high concentrations of CPT-11 and its metabolites. Intra-day analysis was performed with six spiked replicates on 1 day whereas inter-day evaluation was done on 6 separate days. Accuracy was the percent error by which the mean concentration of the replicate samples deviated from the known target concentration (mean concentration/target concentration×100%), and precision was the relative standard deviation (RSD). We also analyzed the accuracy of the HPLC method by assaying three plasma samples prepared by a separate technologist, containing low, medium, and high concentrations all eight analytes which were unknown to the assay operator.

2.7. Recovery

Recovery of the lactone analytes was determined by comparing the peak heights from spiked plasma samples with those from samples prepared with 0.05 *M* citric acid at the same concentration within the validated range. For the carboxylate analytes, the peak heights from spiked plasma samples were compared with those from samples prepared with 0.04 *M* borax. Six sample replicates at low and high concentrations were analyzed (Table 4).

2.8. Linearity and sensitivity

The linearity of the assay procedure was determined by calculation of a regression line using the method of least squares analysis. Concentrations in samples were calculated from the resulting peak heights and the regression equation of the calibration curve. Limit of quantitation was defined as the peak height that was five times larger than baseline noise.

2.9. Collection of patient plasma samples and application of assay

Before CPT-11 was administered, whole blood was collected from a site contralateral to the CPT-11 infusion site into a heparinized tube. Additional samples were collected by the same manner at 0.25,

0.5, 1, 2, 4, and 6 h after the end of a 1-h intravenous (i.v.) infusion of CPT-11 (20 mg/m^2). After collection, the blood was immediately centrifuged at 7000 *g* for 2 min in a microcentrifuge to isolate plasma. Plasma proteins in a volume of 200 μl were precipitated by adding 800 μl of cold (−30 °C) methanol, and vigorous agitation on a vortex mixer. The samples were then centrifuged at 7000 *g* for 2 min. The supernatant was aspirated into a screw-cap tube and stored at −70 °C until analysis.

Plasma concentration–time data of the lactone and total (lactone+carboxylate) forms of CPT-11, SN-38, SN-38G and APC were modeled simultaneously using a Bayesian estimation algorithm with pediatric population priors as implemented in ADAPTII [13,14].

3. Results

3.1. Application of the assay

A chromatogram of a control sample containing all eight analytes is shown in Fig. 2. We detected separate peaks for each analyte (CPT-11 L, CPT-11 C, APC L, APC C, SN-38 L, SN-38 C, SN-38G L, and SN-38G C).

3.2. Calibration curves, linearity, and sensitivity

As shown in Table 1, the calibration curves demonstrated a linear relationship between peak heights and concentration for all eight analytes. The lower limit of quantitation for each analyte was as follows: SN-38 L, 0.5 ng/ml; SN-38 C, 1 ng/ml; APC L, APC C, SN-38G L, and SN-38G C, 2 ng/ml; and CPT-11 L and CPT-11 C, 5 ng/ml.

3.3. Precision, accuracy and recovery

The intra-day (n=6) and inter-day (n=6) precision and accuracy of the assay are summarized in Table 2. At low concentrations, the intra-day RSD was ≤11.5% and the inter-day RSD was ≤18.6% for all eight analytes. At high concentrations of all analytes, the intra-day RSD was ≤15.0% and the inter-day RSD was ≤11.7%. The accuracy ranged from 79.2 to 117.6% at known low and high

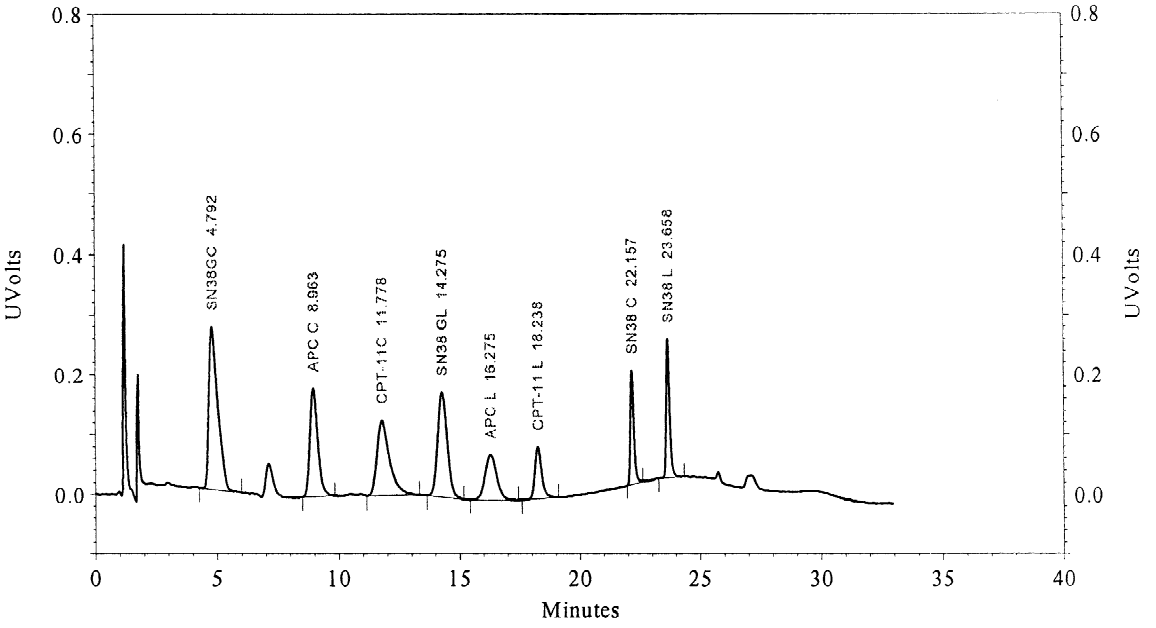

Fig. 2. A representative chromatogram of CPT-11 and its metabolites in a spiked sample to which known concentrations of the lactone and carboxylate forms of CPT-11, SN-38, SN-38G, and APC have been added. The concentration of CPT-11 C and CPT-11 L was 75 ng/ml. The concentration of SN-38 C, SN-38 L, SN-G3G C, SN-38G L, APC C, and APC L was 10 ng/ml.

Table 1
Regression parameters for calibration curve of pooled human plasma spiked with CPT-11 and metabolite standards

	Slope (mean±SD)	*y*-Intercept (mean±SD)	Correlation coefficient (mean±SD)
CPT-11 L	0.00107±0.0001053	−1.67±1.29	0.984±0.015
CPT-11 C	0.00050±0.0001867	−0.36±1.29	0.988±0.011
SN-38 L	0.00019±0.0000852	0.29±0.36	0.961±0.014
SN-38 C	0.00020±0.0000404	−0.05±0.41	0.995±0.002
SN-38G L	0.00062±0.0000493	−1.49±0.15	0.966±0.036
SN-38G C	0.00007±0.0000150	−1.16±0.71	0.961±0.023
APC L	0.00029±0.0000347	−0.65±0.97	0.953±0.033
APC C	0.00012±0.0000122	−1.18±0.54	0.969±0.021

concentrations of all analytes. In experiments in which the analyst did not know the concentrations of analytes added to the plasma sample, the accuracy ranged from 81 to 117% (Table 3).

Mean recoveries from plasma were above 82% for all compounds at low concentrations and above 85.4% for all analytes at high concentrations (Table 4).

3.4. Application of the HPLC assay to the analysis of a patient plasma sample

The assay was suitable for assay of patient plasma. Chromatograms from a patient plasma sample before and at 15 min after the end of a 1-h infusion of CPT-11 (20 mg/m^2) are shown in Fig. 3. Plots of the plasma concentration versus time profile of CPT-11 and its metabolites (lactone and lactone+carboxylate) in a single patient are shown in Fig. 4.

4. Discussion

The results show that our HPLC method permits the simultaneous, accurate, and precise measurement of CPT-11, SN-38, SN-38G, and APC lactone and carboxylate concentrations in human plasma. Only a single run is needed, and the run time is feasible.

Table 2
Precision and accuracy of the HPLC method in measuring known concentrations of the lactone and carboxylate forms of CPT-11 and metabolites added to spiked human plasma

Standard	Target concentration (ng/ml)	Intra-day (n=6)			Inter-day (n=6)		
		Observed concentration (mean±SD)	RSD (%)	Accuracy (%)	Observed concentration (mean±SD)	RSD (%)	Accuracy (%)
CPT-11 L	15	14.24±0.97	6.9	94.9	14.40±1.33	9.2	96.0
	75	72.90±1.62	2.2	97.2	75.45±4.1	5.4	100.6
CPT-11 C	15	17.64±1.66	9.4	117.6	17.16±2.22	12.9	114.4
	75	70.41±3.25	4.6	93.9	67.71±3.35	5.0	90.3
SN-38 L	3	3.08±0.19	6.1	102.8	2.97±0.31	10.4	98.9
	10	10.47±0.11	1.1	104.7	10.23±0.73	7.1	102.3
SN-38 C	3	2.41±0.28	11.5	80.3	2.81±0.52	18.6	93.7
	10	8.38±0.21	2.6	83.8	9.45±1.11	11.7	94.5
SN-38G L	3	2.57±0.27	10.6	85.7	2.71±0.23	8.5	90.2
	10	9.45±0.58	6.1	94.6	9.92±0.55	5.5	99.2
SN-38G C	3	2.93±0.33	11.2	97.6	2.90±0.12	4.1	96.5
	10	9.63±0.49	5.1	96.3	9.64±0.91	9.5	96.4
APC L	3	3.11±0.19	6.0	103.7	3.07±0.16	5.3	102.3
	10	7.92±0.87	11.0	79.2	9.31±0.98	10.5	93.1
APC C	3	2.77±0.30	10.8	92.4	2.78±0.25	9.0	92.9
	10	8.46±1.27	15.0	84.6	9.70±1.11	11.5	97.0

Table 3
Precision and accuracy of the HPLC method in analyzing three replicates of unknown plasma samples spiked with different concentrations of CPT-11 and metabolites[a]

Analyte	Target concentration (ng/ml)	Mean observed concentration (ng/ml)	RSD (%)	Accuracy (%)
CPT-11 L	10	10.01	5.3	100.1
	80	78.24	27.1	97.8
	200	217.85	6.8	108.9
CPT-11 C	10	8.32	13.1	83.2
	80	87.86	8.3	109.8
	200	233.28	1.3	116.6
SN-38 L	3	2.46	5.2	81.8
	12	10.34	6.7	86.2
	20	18.96	1.9	94.8
SN-38 C	3	3.43	5.9	114.2
	12	12.96	6.4	108.0
	20	20.94	1.1	104.7
SN-38G L	3	2.46	3.8	82.1
	12	12.02	3.5	100.1
	20	19.91	9.6	99.6
SN-38G C	3	2.50	12.0	83.4
	12	11.35	5.0	94.6
	20	19.68	2.4	98.4
APC L	3	2.88	4.0	95.9
	12	12.92	12.1	107.6
	20	20.27	9.7	101.4
APC C	3	2.42	4.1	80.9
	12	12.17	1.4	101.4
	20	21.26	7.5	106.3

[a] The analyst was blinded to the sample preparation and the concentrations of analytes added to the plasma.

Moreover, this HPLC method successfully measured the concentrations of the lactone and carboxylate forms of the parent and three major metabolites in the plasma of a patient who received CPT-11.

In developing this method, numerous variables had to be considered. For measurement of the lactone form, a buffer was required in the mobile phase to control the pH. We used 0.75 *M* ammonium acetate at a pH of 6.0 as our buffer for the mobile phase. To simultaneously measure the lactone and

Table 4
Mean recovery of irinotecan at low ($n=6$) and high ($n=6$) concentrations of analytes

Analyte	SN-38G C	APC C	CPT-11 C	SN-38G L	APC L	CPT-11 L	SN-38 C	SN-38 L
Low concentration								
Concentrations (ng/ml)	3	3	15	3	3	15	3	3
Mean recovery (%)	82	89.3	105	117.23	87.5	92.8	88.9	91.3
RSD (%)	1.12	0.66	0.82	1.45	0.59	0.48	0.74	0.88
High concentration								
Concentrations (ng/ml)	10	10	75	10	10	75	10	10
Mean recovery (%)	103.9	95	96.1	85.4	99.1	101.8	99.1	89.9
RSD (%)	1.7	0.96	0.59	0.89	3.67	1.32	0.5	0.45

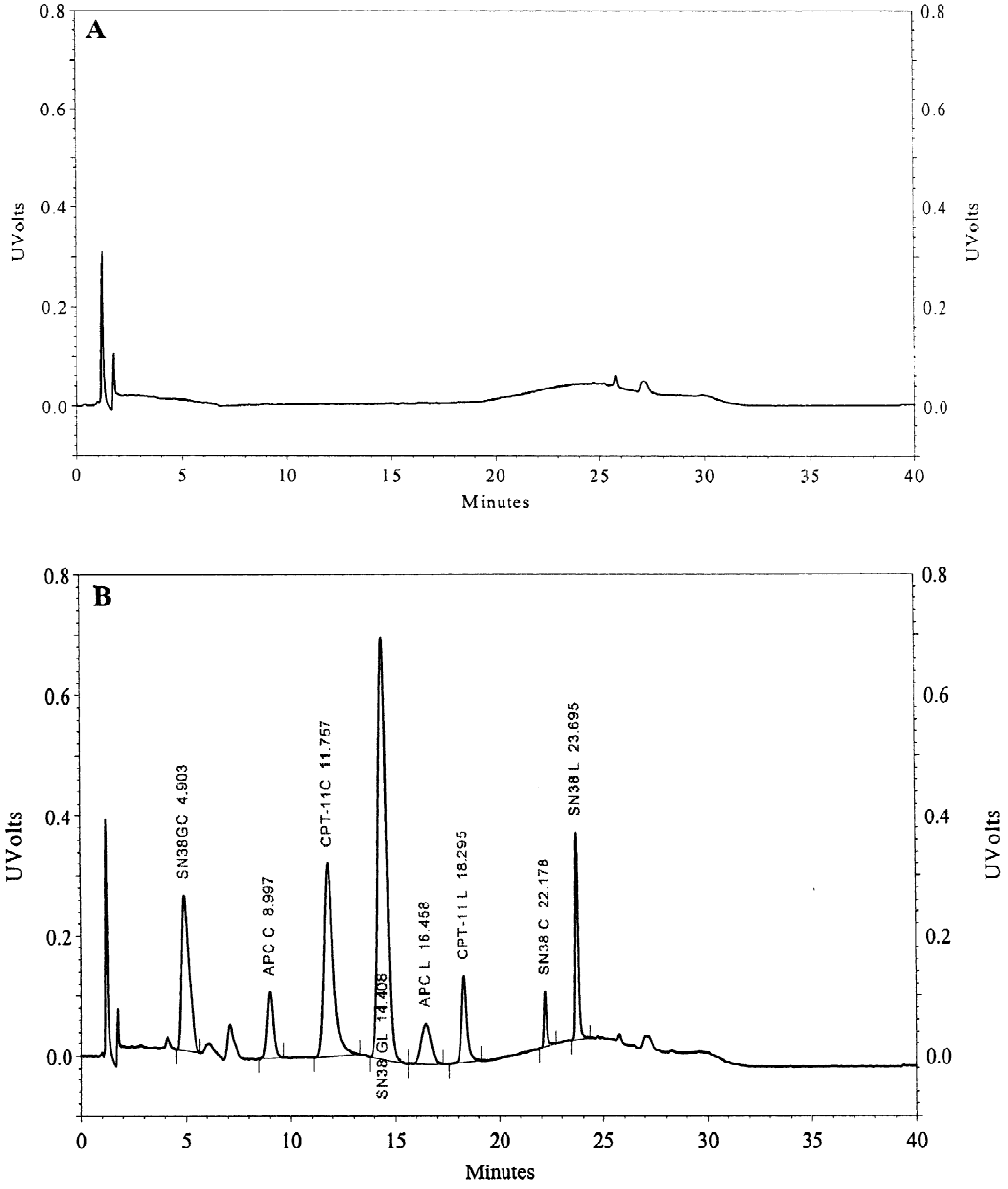

Fig. 3. Chromatograms of a patient's plasma samples drawn before the infusion of CPT-11 (panel A) and 15 min after the end of the 1-h intravenous infusion of 20 mg/m^2 (panel B).

carboxylate species, we used the ion-pairing agent tetrabutylammonium phosphate to increase the affinity of the carboxylate species for the column, so that they were not eluted with the void volume. Acetonitrile was added to the buffer to control retention of the lactone analytes on the column. A mobile phase gradient was used to decrease the retention times of the carboxylate and lactone forms of SN-38, which were the last two analytes to be eluted from the column. To increase the sensitivity of the assay, we optimized the peak height and shape for each compound by individualizing the excitation wave-

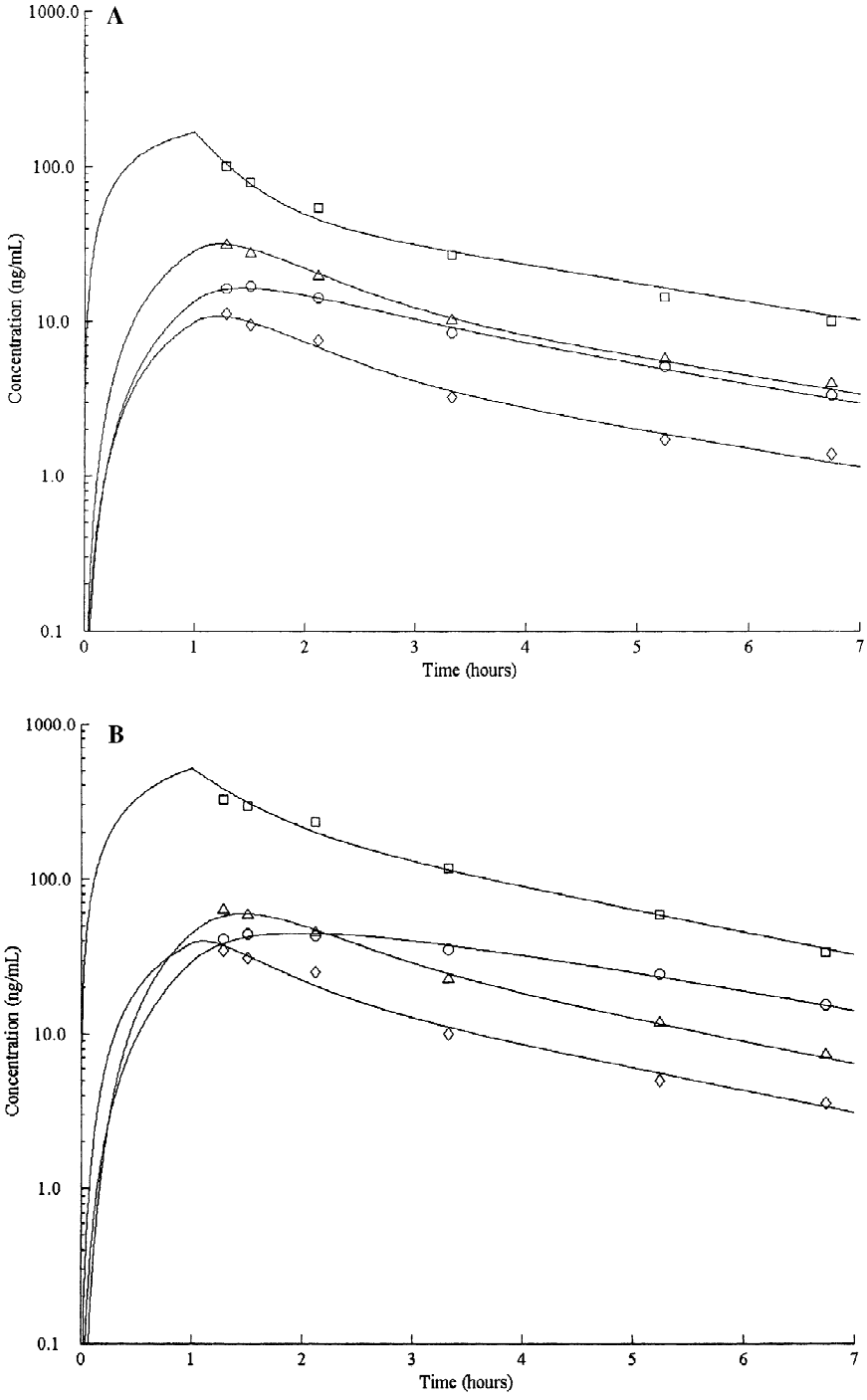

Fig. 4. Concentration versus time plot of CPT-11 and metabolites in a single patient after a 1-h intravenous infusion of irinotecan 20 mg/m^2 per day. Symbols indicate measured plasma irinotecan (□), SN-38G (△), APC (○), and SN-38 (◊) lactone data (panel A) and total (lactone+carboxylate) data (panel B). Lines represent the best-fit curves using a Bayesian estimation algorithm for a first-order structural pharmacokinetic model to simultaneously fit the parent drug and metabolite data.

length specifically for each analyte. The use of an internal standard was not necessary because the linearity of the calibration curves ($r^2 \geq 0.95$) was acceptable and the intra-day and inter-day precision of the assay in measuring control samples without internal standards was adequate. Although all metabolites were fully resolved by 25 min, a run time of 40 min was used. This extended run time ensured a more stable baseline for the start of the next auto injection.

The pharmacokinetic–pharmacodynamic relationships of CPT-11 and its metabolites, including drug interactions and phenotype–genotype correlations with promoter polymorphisms of the uridine diphosphate glucuronosyltransferase enzyme UGT1A1, are still being characterized in clinical studies [15–17]. The extent of SN-38 glucuronidation in individual patients has been shown to be related to toxicity [3]. Likewise, the systemic exposure of the metabolite APC serves as an important marker for the oxidative metabolism of the parent drug [10]. Our HPLC method permits the simultaneous measurement of the lactone and carboxylate forms of CPT-11, SN-38, SN-38G, and APC in a single run, allowing flexibility to study various aspects of the pharmacokinetics and pharmacodynamics of CPT-11. Our method provides advantages over previously published methods which require multiple injections [3,7] or which cannot measure lactone and carboxylate species separately [8,9]. This method has provided the selectivity and sensitivity needed to fully describe the pharmacokinetic profiles of CPT-11 and its three major metabolites in patients receiving CPT-11.

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