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ORIGINAL RESEARCH

An analytical method for irinotecan (CPT-11) and its metabolites using a high-performance liquid chromatography: parallel detection with fluorescence and mass spectrometry

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ABSTRACT: Irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) is an anticancer pro-drug used in the treatment of many types of cancer. We describe here the validation of an analytical method for CPT-11 and its metabolites, including an active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), its glucuronidated form, SN-38G, and several cytochrome P450 3A-mediated products such as 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) using a high-performance liquid chromatography connected to parallel fluorescence and mass spectrometry detection systems. This method is characterized as follows: (1) simple extraction of the analytes from biomaterials with perchloric acid/methanol; (2) sensitive quantitation of major metabolites (SN-38G, SN-38 and APC) with a fluorescence detector (FLD), where the limits of quantitation by FLD were 2.5 ng/mL for SN-38G and APC, 5 ng/mL for CPT-11 and 1 ng/mL for SN-38, respectively; (3) parallel selective monitoring of the metabolites including minor metabolites with a mass selected detector (MSD). There was no observed interference by other drugs expected to be co-administered. This method showed its usefulness by identifying a novel metabolite produced in human hepatic microsomes. The results indicate that this combination of FLD and MSD enables a highly selective analysis of CPT-11 and its metabolites, and is useful for studies both *in vivo* and *in vitro*. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Irinotecan or 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy camptothecin (CPT-11), an anticancer pro-drug, is a water-soluble derivative of camptothecin, a plant alkaloid isolated from the Chinese tree, *Camptotheca acuminuta*. CPT-11 is currently used for the treatment of colon cancer (Rougier and Bugat, 1996), lung cancer (Negoro *et al.*, 1991), as well as other types of cancers (Saijo, 1996). An active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38) which acts as an inhibitor of topoisomerase I (Topo I; Hsiang and Liu, 1996; Hertzberg *et al.*, 1989) is produced after enzymatic cleavage by carboxylesterase(s) (CES) in the liver (Slatter *et al.*, 1997; Humeruckhouse *et al.*, 2000), small intestine (Khanna *et al.*, 2000), and plasma (Kehrer *et al.*, 2000). SN-38 is further conjugated by uridine diphosphate glucuronosyl transferase (UGT) 1A1 in the liver to yield SN-38 glucuronide (SN-38G; Iyer *et al.*, 1998), which is excreted in the urine and bile (Lokiec *et al.*, 1996).

Other metabolic pathways for CPT-11 are oxidation reactions mediated by cytochrome P-450 (CYP) 3A. Major oxidation products catalyzed by CYP3A4 are 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin (APC) (Rivory *et al.*, 1996; Haaz *et al.*, 1998) and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (NPC; Dodds *et al.*, 1998; Haaz *et al.*, 1998), both of which are weak inhibitors of Topo I. Other CYP3A-mediated metabolites, such as

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Abbreviations used: APC, 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; CES, carboxyl esterase; FLD, fluorescence detector; MSD, mass selected detector; SN-38, 7-ethyl-10-hydroxycamptothecin; Topo I, topoisomerase I; UGT, uridine diphosphate glucuronosyl transferase.

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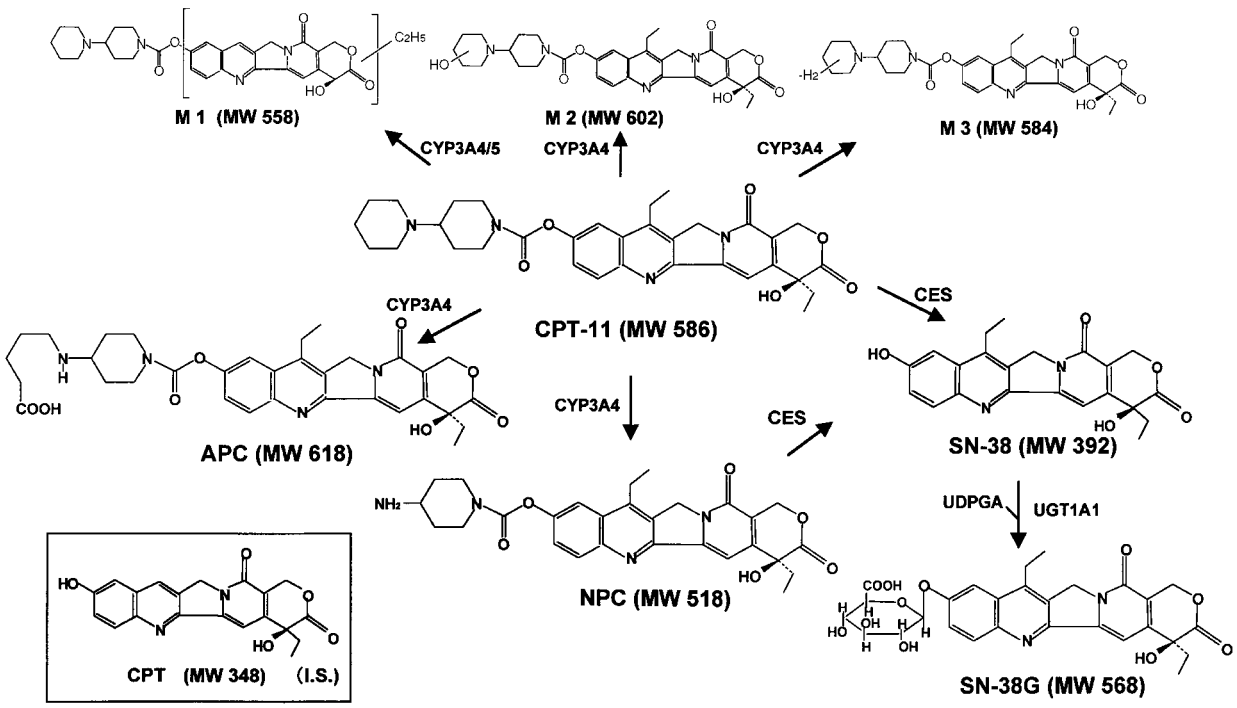

Figure 1. Structures of CPT-11 metabolites and established metabolic pathways. CPT was used as internal standard (IS). Exact positions of a hydroxyl group in M1 and a double bond in M3 are not defined.

hydroxylated (Lokiec *et al.*, 1996), de-ethylated (Santos *et al.*, 2000) and dehydrogenated (Sai *et al.*, 2001) metabolites, have been detected in human liver microsomes; however, their biological actions or metabolic fates in humans have not yet been clarified (Fig. 1).

With the extensive pharmacokinetic studies on CPT-11 in patients, sensitive analytical methods for CPT-11 metabolites, especially for SN-38 and SN-38G, have been developed using high-performance liquid chromatography (HPLC) (de Bruijin *et al.*, 1999; Escoriaza *et al.*, 2000; Rogot *et al.*, 1999; Rivory *et al.*, 1999; Loos *et al.*, 2000). Recently, a variety of CYP3A4-mediated metabolites have been detected (Lokiec *et al.*, 1996; Slatter *et al.*, 2000; Santos *et al.*, 2000). Methods for the detection of the minor metabolites including NPC, de-ethylated (M1), hydroxylated (M2) and dehydrogenated (M3) metabolites, however, need to be improved and validated.

In the present paper, a highly selective analytical method was developed and validated to detect CPT-11 and its major metabolites, including SN-38, SN-38G and APC, and to identify its various minor metabolites, including NPC, M1, M2 and a novel metabolite, M3. We employed an HPLC system connected to parallel detectors; one is a sensitive detection system by fluorescence detector (FLD) and another is a selective monitoring system by mass selected detector (MSD). This combined detection enabled highly selective analysis of CPT-11 and its metabolites. The usefulness of this method for *in vitro* study is also described.

EXPERIMENTAL

Chemicals. CPT-11 (lot no. 115122), SN-38 (lot no. 970507R), APC (lot no. 970730), NPC (lot no. 000914), and SN-38G (lot no. 970507R) were kindly supplied by Yakult Honsha Co. Ltd (Tokyo, Japan). (*S*)-(+)-Camptothecin (CPT) was purchased from Tokyo Kasei Co. Ltd (Tokyo, Japan). Pooled human hepatic microsomes (HG161) were obtained from Gentest Corporation (Woburn, MA). β-NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and uridine 5′-diphosphoglucuronic acid (UDPGA) were obtained from Sigma Chemical Co. (St Louis, MO). Acetonitrile, methanol and ammonium acetate were reagent grade and obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of standard solutions. CPT-11, SN-38G, SN-38 or APC was dissolved in DMSO at concentrations of 1 mg/mL for stock solution of the standards. Mixed stock solution of the four standards was prepared by diluting the stock solution of CPT-11, SN-38G, SN-38 and APC to final concentrations of 450, 100, 10, 100 μg/mL, respectively, in DMSO. Aliquots of the mixed stock solution were stored in polyethylene tubes at −80°C. Working solutions were prepared daily by sequential dilution of the mixed standards solution in 0.01 N HCl:methanol (2:3) to final

concentrations of 3.6, 18, 72 and 144 μg/mL for CPT-11, 0.8, 4, 16 and 32 μg/mL for SN-38G and APC, and 0.08, 0.4, 1.6 and 32 ng/mL for SN-38. Standard solutions for calibration curves were prepared in duplicate by addition of 25 μL of the working solution into 975 μL of drug-free human plasma to make concentrations of 90, 450, 1800 and 3600 ng/mL for CPT-11, 20, 100, 400 and 800 for SN-38G and APC, and 2, 10, 40 and 80 ng/mL for SN-38. CPT as an internal standard (IS) was dissolved in DMSO at a concentration of 105 ng/mL and stored at −80°C. The working IS solution was made daily by diluting the stock in a 50-fold volume of 0.01 N HCl:methanol (2:3).

Preparation of analytical samples from human plasma. Frozen plasma samples were thawed in a water bath at room temperature for 10 min and then kept on ice. Two hundred microliter aliquots of thawed plasma were transferred to polypropylene microtubes and mixed with 200 μL of methanol/5% perchloric acid (1:1) containing 10 μL of IS (final concentration of 100 ng/mL) and mixed for 3 min, followed by centrifugation at 14 000***g*** for 3 min. The supernatant (200 μL) was mixed with 40 μL of 5 M ammonium acetate buffer (pH 4.5) and transferred to an analytical vial for HPLC/MS after filtration with a filter cartridge (0.45 μm; Millipore). The prepared samples were kept in an autosampler at 4°C until injection.

Incubation of CPT-11 with human hepatic microsomes. A typical reaction mixture in a total volume of 200 μL contained 1 mg protein/mL of pooled human hepatic microsomes, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, 5 mM UDPGA, and 10 μM CPT-11 in 100 mM potassium phosphate buffer (pH 7.4). Reactions were incubated at 37°C for 10 min. The reaction was terminated by the addition of 200 μL of methanol/5% perchloric acid (1:1) containing 10 μL of CPT (IS). Further preparation of analytical samples was the same as described for the human plasma samples.

HPLC conditions. For the analysis of CPT-11 and its metabolites, an HP 1100 model HPLC system equipped with FLD (G1321A) and with MSD (G19146A; Hewlett Packard, Les Ulis, France) was used. Chromatographic separation was performed using an analytical column of CAPCELL PAK CN UG120, S-5 μm (4.6 mm i.d. ×150 mm; Shiseido, Co. Ltd, Tokyo, Japan) protected by a NewGuard C8 column (Perkin-Elmer Co., Norwalk, CT, USA) at a temperature of 40°C. The mobile phase for separation consisted of a mixture of acetonitrile:methanol:0.05 M ammonium acetate (pH 4.5; 14:14:72) and was delivered at a flow rate of 0.5 mL/min. For the detection system, a parallel setting of FLD and MSD was adopted instead of a tandem connection of two detectors, since an analytical cell in FLD is not durable to the back-pressure coming from MSD. The column outlet was split into two paths—one directed to a fluorescence detector and the other connected to a mass spectrometer at a 3:1 tube diameter ratio, ie approximately 9:1 volume ratio. For fluorescence detection, the excitation wavelength was set at 368 nm, and the emission wavelengths were at 432 nm for CPT-11, SN-38G, APC and NPC and at 535 nm for SN-38. MS analysis by the atmospheric pressure ionization-electrospray mode was used for identification of several metabolites of CPT-11. The selected ions monitored as protonated molecules (MH^+) were as follows: *m/z* 569 for SN-38G, *m/z* 559 for M1, *m/z* 519 for NPC, *m/z* 603 for M2, *m/z* 619 for APC, *m/z* 585 for M3 (new metabolite), *m/z* 587 for CPT-11, *m/z* 349 for CPT, and *m/z* 393 for SN-38. Nitrogen was used as the nebulization and curtain gas, and was delivered at a flow rate of 7 L/min at a temperature of 250°C. The nebulizer pressure was 45 psig, the capillary voltage was 4000 V, and the fragmentation voltages were set at 400 V for SN-38G and 120 V for all other ions. Detection and integration of chromatographic peaks were performed by the HP Chemistation data analysis system (Hewlett Packard, Les Ulis, France). The peak area was used as a response.

Stability tests. To evaluate the stability of CPT-11 and its metabolites in human plasma during long-term storage at −80 °C, two different spiked plasma samples were prepared. Concentrations for the low dose sample (L-dose) were 200 ng/mL for CPT-11, 40 ng/mL for SN-38G and APC, and 4 ng/mL for SN-38, and those for the high dose sample (H-dose) were 4000 ng/mL for CPT-11, 400 ng/mL for SN-38G and APC, and 40 ng/mL for SN-38. Aliquots of these samples were stored at −80°C and analysis in duplicate was performed every 2 weeks up to for 8 weeks. Adsorption of spiked compounds to storage containers during storage at −80°C was tested using glass, polypropylene and siliconized tubes. A mixture of standards was dissolved in phosphate buffered saline (pH 7.4) at concentrations of 500 ng/mL for CPT-11, 100 ng/mL for SN-38G and APC, and 10 ng/mL for SN-38. An aliquot was distributed into each of three types of containers and kept at −80°C for up to 8 weeks. To test the stability after repeated freeze and thaw cycles, a spiked plasma sample was prepared at concentrations of 100 ng/mL for SN-38G and APC, 500 ng/mL for CPT-11 and 10 ng/mL for SN-38. The mixed sample was stored at −80°C for at least 6 h per cycle, and thawed at room temperature for 10 min and then kept on ice for 30 min before re-freezing. The freeze–thaw cycle was repeated five times. Duplicate samples were analyzed before freezing and after three and five cycles. Changes in the amounts were evaluated by comparison with duplicate reference samples at the same concentration prepared fresh. To examine short-term stability, spiked samples were prepared separately in polypropylene tubes using plasma obtained from three healthy volunteers at concentrations of 100 ng/mL for SN-38G and APC, 500 ng/mL for CPT-11 and 10 ng/mL for SN-38. The spiked samples were kept on ice and the duplicates for each specimen were determined at 0, 1 and 2 h.

Selectivity. Blank plasma samples were provided from six healthy volunteers and those chromatograms were compared with one obtained from standard solutions. Possible interference on HPLC-chromatograms of CPT-11 metabolites was tested with regard to 18 drugs (5 μg/mL each) may be co-administered to patients along with CPT-11.

Validation. The detection of the standards in plasma was determined at 20 and 800 ng/mL for SN-38G and APC, 90 and 3600 ng/mL for CPT-11, 2 and 80 ng/ml for SN-38, and 100 ng/mL for CPT (IS) in quintuple analyses. The means of the fluorescence area intensity were obtained from quintuple spiked samples in plasma and distilled water. Percentage of the mean area obtained from spiked plasma against that from distilled water was defined as recovery. For the accuracy and precision test, the stock solution of standards was diluted in human plasma to obtain two levels of quality control (QC). Final concentrations of the standards in a

Table 1. Stability of CPT-11 metabolites in human plasma during storage at −80°C

Chemical	Added (ng/mL)	Storage time (weeks)				
		0	2	4	6	8
SN-38G	40	100	102	92.7	91.1	83.3
	400	100	105	104.8	100.2	99.4
APC	40	100	101	90.5	89.3	84.4
	400	100	103	102.1	99.9	99.4
CPT-11	200	100	100	89.4	88.3	82.8
	2000	100	100	99.4	97.8	97.6
SN-38	4	100	100	83.8	88.7	84.3
	40	100	100	99.2	96.4	96.1

Each value is the mean from duplicate samples and expressed as percent of data on 0 weeks.

lower dose for QC (QC-L) and in a higher dose for QC (QC-H) were as follows: QC-L—10 ng/mL of CPT-11, SN-38G and APC, and 2 ng/mL of SN-38); QC-H—3000 ng/mL of CPT-11, 500 ng/mL of SN-38G and APC, and 80 ng/mL of SN-38. Aliquots of the plasma samples were stored at −80°C in polyethylene tubes. The duplicate samples were determined on each day for 6 days (one-way layout). Intra-day and inter-day precision (repeatability and intermediate precision) were calculated according to the method described in ISO5725-2 (IOS, 1994). For the limit of quantitation (LOQ) analysis, stock standards were mixed in 0.01 N HCl:methanol (2:3) at a concentration of 50 μg/mL each, and diluted in plasma to obtain LOQ-samples, where a range of final concentrations for each standard was from 1 to 5 ng/mL. Precision (RSD) and accuracy (relative standard error; RSE) were calculated by determining six replicates for each concentration. LOQ was defined as the lowest concentration with an acceptable limit of 20% for both precision and accuracy (Shah *et al.*, 1991).

Application of CPT-11-calibration curve to determination of its metabolites. A ratio of the slope for each standard curve against that for CPT-11 was calculated for data from six experiments and the mean value of the slope-ratio to CPT-11 for each standard was obtained (i). Determination of CPT-11 metabolites was undertaken using the slope-ratio to CPT-11 (f_m; a constant for each metabolite) and a CPT-11-calibration curve obtained in each run (ii). Precision for the estimated concentrations of the metabolite using f_m and the accuracy against the values obtained using calibration curves were evaluated on data from six experiments.

Formula of calibration curve for CPT-11

$$y_{\text{CPT-11}} = a_{\text{CPT-11}} \cdot x_{\text{CPT-11}}$$

Formula of calibration curve for a metabolite

$$y_m = a_m \cdot x_m$$

Slope ratio to CPT-11 of calibration curve

$$f_m = 1/N\Sigma(a_m/a_{\text{CPT-11}}). \quad \text{(i)}$$

Estimated concentration of metabolite

$$X_m = 1/(a_{\text{CPT-11}} \cdot f_m) \cdot Y_m. \quad \text{(ii)}$$

where y-area ratio of a standard to IS, x-concentration of a standard, Y-observed area ratio of an analyte to IS, X-estimated concentration of an analyte, a-slope of the calibration curve, m-SN-38G, APC or SN-38 and $N = 6$.

RESULTS

Stability of CPT-11 and its metabolites

In a preliminary test, we found that the treatment of spiked plasma samples with perchloric acid/methanol and incubation at room temperature facilitated degradation of the extracted standards. However, adjustment of the pH to 4.5 or storage at 4°C prevented the degradation of the extracted standards for at least 24 h. Based on this finding, the pH of the extracts was adjusted to 4.5 by adding ammonium acetate buffer and temperature of the autosampler was maintained at 4°C.

Various parameters that might affect short-term stability were also tested. After five freeze–thaw cycles of the spiked samples, the changes in levels of SN-38G, APC, CPT-11 and SN-38 were all within 10%. The spiked standards in human plasma kept on ice after freeze–thawing did not change for at least 2 h, enough time to prepare the analytical samples for each run. There was no significant difference between containers, ie glass, polypropyrene or siliconized tubes, in the relative changes of the standards dissolved in PBS for the 6 weeks' storage period at −80°C (data not shown), suggesting any of the containers tested are suitable for long-term storage of clinical samples. Polypropylene tubes were used in subsequent studies.

Results of long-term stability of the standards kept at −80°C are summarized in Table 1. The amounts of SN-38G, SN-38, APC and CPT-11 at higher concentrations did not change at least for 8 weeks. At lower concentrations, the standards showed a tendency to decrease by approximately 10% after 6 weeks of storage. These results suggested that analysis of CPT-11 metabolites in

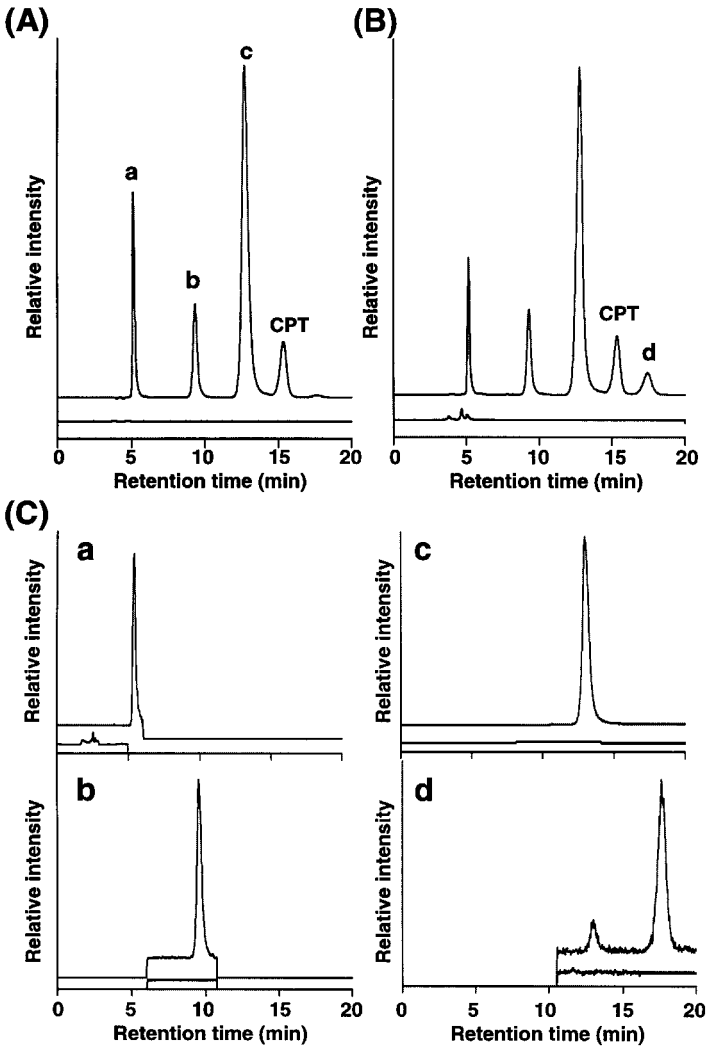

Figure 2. Typical HPLC chromatograms of CPT-11 and its metabolites spiked in human plasma. (A) Fluorescent signal at 368 nm of excitation and at 432 nm of emission. (B) Fluorescent signal at 368 nm of excitation and at 535 nm of emission. (C) Selected ion signals monitored by MSD. Protonated mass molecules (MH^+) monitored were as follows: (a) *m/z* 569 for SN-38G; (b) *m/z* 619 for APC; (c) *m/z* 587 for CPT-11; (d) *m/z* 393 for SN-38. CPT was used as an IS. Chromatogram for blank plasma is shown under that of spiked plasma in each panel.

frozen plasma samples should be undertaken within 1 month.

HPLC chromatogram

For extraction of standards from the plasma, we first tried organic solvent treatment with acetonitrile, followed by incubation with acetic acid. However, broad interference around the CPT-11 peak appeared on the HPLC chromatogram. Such interference was avoided with the application of perchloric acid/methanol as an extraction solvent according to a method by Sparreboom *et al.* (1998). A typical HPLC-chromatogram is shown in Fig. 2. In the present analytical method, a CN-column and a uniphasic HPLC mobile phase were applied according to the method by Slatter *et al.* (2000), with a slight modification of the composition of mobile phase, ie acetonitrile:methanol:0.05 N ammonium acetate (pH 4.5) = 1.4:1.4:7.2, to obtain resolution of more than 1.0 for all the determinants. For FLD, two levels of emission wavelength were set for each run to obtain the maximum signal intensity for the determinants; at 432 nm for SN-38G, APC and CPT-11 [Fig. 2(A)], and at 535 nm for SN-38 [Fig. 2(B)], respectively. Simultaneous detection of metabolites with SIM by MSD was employed to identify all the determinants [Fig. 2(C)]. Retention times of each

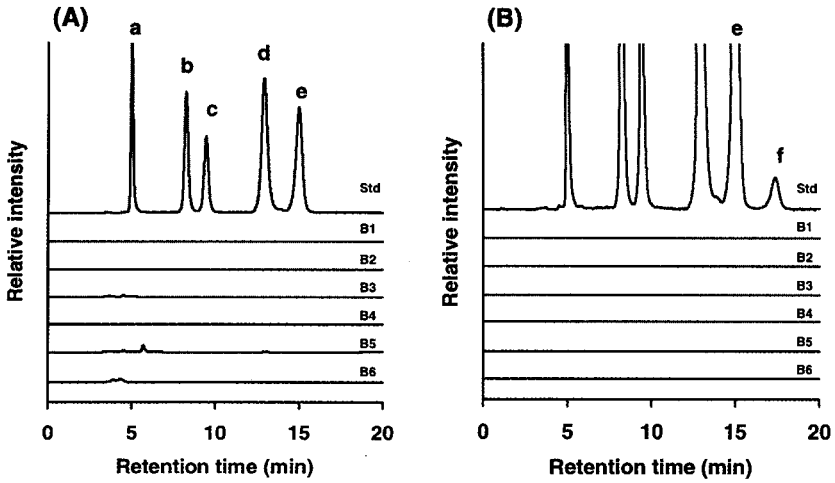

Figure 3. Selectivity for detection of CPT-11 metabolites. (A) Fluorescent signal at 368 nm of excitation and at 432 nm of emission. (B) Fluorescent signal at 368 nm of excitation and at 535 nm of emission. Blank plasma samples were provided from six healthy volunteers and those chromatograms (B1–6) were compared with those of the spiked plasma (Std). Concentrations of standards correspond to 40-fold LOQ values; 100 ng/mL for SN-38G (a), NPC (b) and APC (c), 200 ng/mL for CPT-11 (d), and 40 ng/mL for SN-38 (f). CPT (e) was used as IS.

standard in FLD and MSD were 5.1 and 5.4 min for SN-38G, 9.6 and 9.9 min for APC, 13.3 and 13.6 min for CPT-11, 15.2 and 15.5 min (data for MSD not shown) for CPT (IS), and 17.4 and 17.8 min for SN-38, respectively. In the MSD chart for SN-38, a smaller peak between 13–14 min, which is derived from fragmentation of CPT-11, was observed [Fig. 2(C-d)].

Selectivity

Chromatograms of blank plasma samples obtained from six different persons and of spiked standards in the pooled human plasma are shown in Fig. 3. There were no interfering peaks on the chromatograms obtained from the six individuals, and background signals were smaller than those of 10% of LOQ for all the standards. None of the signals were detected in the chromatograms in FLD or MSD obtained from the 18 drugs (cisplatin, docetaxel, etoposide, 5-fluorouracil, uracil, paclitaxel, vinorelbine, dexamethasone, diclofenac domperidone, granisetron, loxoprofen, mefenamic acid, metoclopramide, naproxen, morphine and codeine), which are potentially co-administered with CPT-11 (data not shown).

Recovery

Recoveries of standards spiked in the plasma were determined for two concentrations covering the expected concentrations in the plasma of patients. There were slight decreases in recovery for the higher concentrations compared with that for the lower concentrations of all the standards. The complete recovery observed for SN-38G may be attributed to its high hydrophilicity. The apparent higher recovery for SN-38G at a low concentration may be due to the background noise, but this did not affect the quantitation of SN-38G, where its concentration is 50 times higher than LOQ. Nearly 80% of recoveries were obtained for less hydrophilic compounds, APC, CPT-11 and SN-38 (Table 2).

Validation

For setting the calibration curves, three weighting functions, ie 1, $1/y$ and $1/y^2$, were investigated, and a function of $1/y$ was selected due to the highest correlation coefficient (r^2). Ranges of linearity in FLD were 2.5–800 ng/mL for SN-38G ($r = 0.996$) and APC ($r = 0.999$), 5–4000 ng/mL for CPT-11 ($r = 0.999$) and 1–80 ng/mL

Table 2. Recovery of CPT-11 and its metabolites determined by HPLC/FLD

Chemical	Added (ng/mL)	Recovery (%)	Added (ng/mL)	Recovery (%)
SN-38G	20	114.7	800	101.0
APC	20	96.6	800	82.5
CPT-11	90	81.8	3600	78.2
CPT	100	62.9	100	62.4
SN-38	2	87.3	80	84.7

Recovery is defined as percent of the mean value of fluorescent signal (area) from quintuplet spiked plasma samples against the value from spiked distilled water.

Table 3. Intra-day and inter-day variation of determination of CPT-11 metabolites by HPLC

		FLD		MSD	
Chemical	Added (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
SN-38G	10	2.02	3.07	22.3	20.3
	500	3.55	3.70	9.30	10.9
APC	10	3.06	2.91	8.45	12.3
	500	3.01	3.19	3.55	4.88
CPT-11	10	4.73	5.21	7.96	9.86
	3000	2.59	2.80	3.22	4.93
SN-38	1	5.67	4.82	6.79	14.75
	80	3.03	3.41	3.55	6.80

A set of analysis in duplicate samples was performed each day for 6 days.

for SN-38 ($r = 0.997$), respectively. In case of MSD, ranges of linearity were 10 to 800 ng/ml for SN-38G ($r = 0.997$) and APC ($r = 0.999$), 10–4000 ng/mL CPT-11 ($r = 0.999$) and 2–80 ng/mL for SN-38 ($r = 0.985$).

The results of a precision test are shown in Table 3. Both the intra-day and inter-day variations were less than 6% at the lower concentration and less than 4% at the higher concentration in the FLD system (Table 3). The small values obtained in the precision test indicated that this method is highly reproducible and satisfactory as a routine analytical method in pharmacokinetic studies. The result is also compatible with the precision data of previous reports (de Bruijin *et al.*, 1999; Escoriaza *et al.*, 2000; Ragot *et al.*, 1999; Sparreboom *et al.*, 1998). Likewise, the MSD system showed precisions in both intra-day and inter-day testing within 10% for all the chemicals at the higher concentration, and within 7–22% at the lower concentration. The result of LOQ determined with FLD is summarized in Table 4. The values obtained for all the chemicals indicate that this method is sensitive enough to determine the clinical levels of CPT-11 metabolites (de Bruijin *et al.*, 1999; Escoriaza *et al.*, 2000; Sparreboom *et al.*, 1998). The values of LOQ determined by MSD were greater than those determined with FLD 2–4-fold; ie 10 ng/mL for SN-38G, APC and CPT-11, and 2 ng/mL for SN-38. This result may be attributed to the general property of detection systems. In MSD system, only a part of sample sprayed is introduced to the quadropole and therefore the volume of sample reaching the detector is variable. This might result in inferior precision observed in MSD to that in FLD, especially at lower concentrations. Therefore, FLD might be more suitable for the quantitation of CPT-11 metabolites in which concentrations are very low. For the analysis of APC, which is expected to be a major product in the clinical samples, the MSD system is also suitable with satisfactory precision, in case peaks of other metabolites might overlap the APC-peak.

Application of CPT-11 calibration-curve for its metabolites

We tested another quantitation method of major metabolites using only a CPT-11 calibration curve to avoid consuming valuable reference standards other than CPT-11. Repetition of our calibration experiments revealed that the slope-ratio of each standard curve to CPT-11 is constant between each experiment, and it is expected that quantitation of its metabolites may be possible using the slope-ratio to CPT-11 (constant) and a calibration curve of CPT-11 obtained in each experiment. Table 5 shows the mean value of the slope-ratio of each standard to CPT-11 determined by FLD. Precision for the concentrations estimated using the slope ratio and their accuracy relative to the values obtained using their own calibration curves were evaluated with data from six experiments (Table 6). Both the intra-day and inter-day precisions were within 5% except at lower concentration of SN-38 (7%). Accuracy was within 0.5% for all the chemicals. These results suggested that use of the CPT-11 calibra-

Table 4. Limit of quantitation (LOQ) of CPT-11 and its metabolites by HPLC/FLD

Chemical	LOQ (ng/ml)	Precision RSD (%)	Accuracy RSE (%)
SN-38G	2.5	9.59	17.4
APC	2.5	8.52	13.7
CPT-11	5	3.25	18.7
SN-38	1	8.42	1.61

Each value was obtained by determination of six replicates.

Table 5. Slope-ratio of calibration curve of each standard to CPT-11 (FLD)

Metabolite	Ratio
SN38-G	1.592
APC	1.135
CPT-11	1.00
SN-38	4.878

Calculation was performed using data from six calibration curves for each standard.

Table 6. Precision and accuracy of determination of CPT-11 metabolites calculated by calibration-curve ratio to CPT-11 (FLD)

Chemical	Added (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy RSE (%)
SN-38G	10	2.54	4.15	0.47
	500	3.58	3.73	−0.44
APC	10	2.76	4.22	−0.16
	500	3.01	3.18	0.31
SN-38	1	7.09	7.50	0.31
	80	3.03	2.94	0.43

Calculation was performed using data on Table 3.

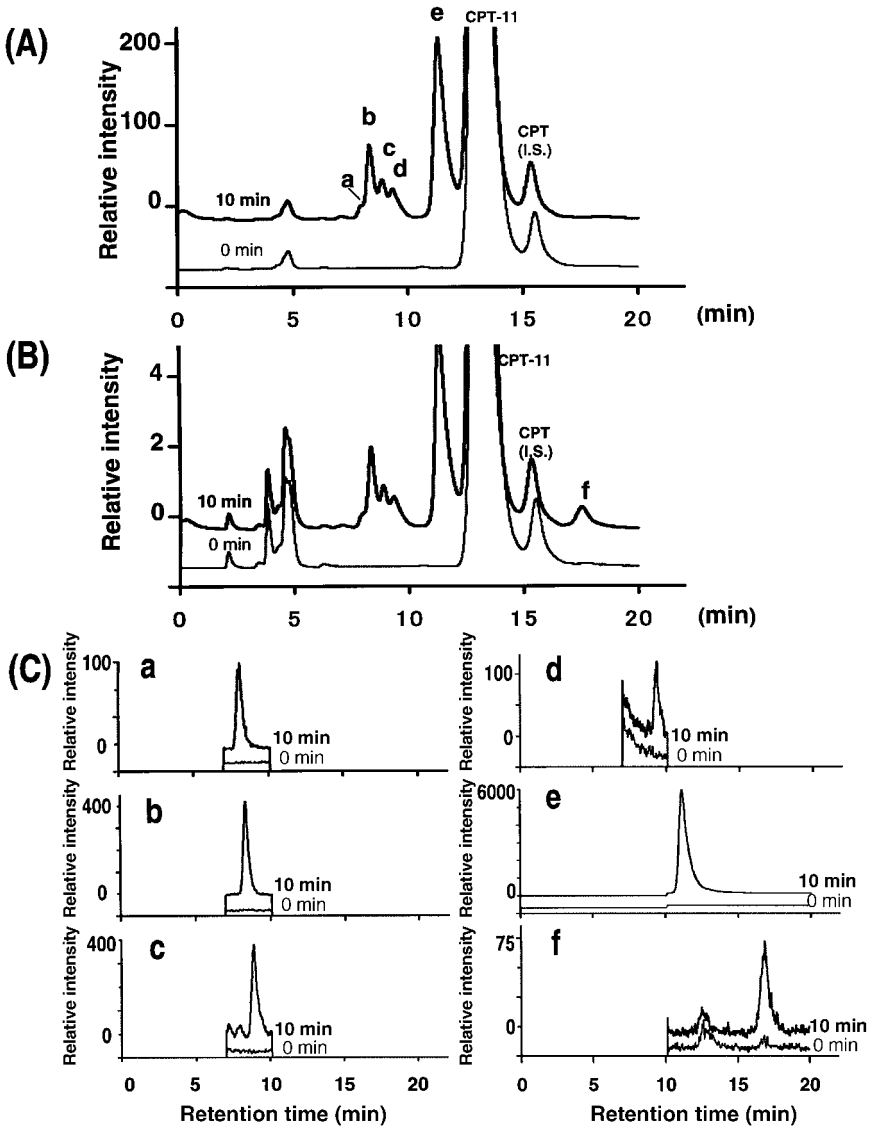

Figure 4. Representative HPLC-chromatograms of CPT-11 metabolites obtained after incubation of 10 μM CPT-11 with human hepatic microsomes (1 mg protein/mL) at 37°C for 10 min. (A) Fluorescent signal at 368 nm of excitation and at 432 nm of emission. (B) Fluorescent signal at 368 nm of excitation and at 535 nm of emission. (C) Selected ion signals monitored by MSD. Protonated mass molecules (MH^+) monitored were as follows: (a) *m/z* 559 for M1; (b) *m/z* 519 for NPC: (c) *m/z* 603 for M2; (d) *m/z* 619 for APC; (e) *m/z* 585 for M3; (f) *m/z* 393 for SN-38. CPT was used as IS. Chromatograms for 0 time and 10 min incubations are shown by thin and thick lines, respectively.

tion curve to quantify its metabolites might be possible in order to avoid consuming other standards or in case it was difficult to obtain them.

Analysis of CPT-11 metabolites produced in the human hepatic microsomes

To examine selective separation of CPT-11-metabolites, including minor products, with our detection system, we analyzed CPT-11-metabolites produced in human liver microsomes. Figure 4 shows a representative HPLC-chromatogram with FLD (A and B) and MSD (C) of CPT-11-metabolites obtained in the microsomal system. Several peaks were detected in the chromatograms including CYP3A4-mediated products, such as NPC, APC and M2 (a hydroxylated product) and a reportedly CYP3A5-mediated product, M1 (a de-ethylated product). In the pharmacokinetic studies on patients treated with CPT-11, APC was reported to be a major CYP3A4-mediated metabolite, but NPC is a minor one (Kehrer *et al.*, 2000; Slatter *et al.*, 2000; Sparreboom *et al.*, 1998). In case of the human microsomal system, NPC and M2 were more dominant metabolites than APC as reported previously (Dodds *et al.*, 1998). More importantly, a novel product, designated as M3, was detected as the most intensive peak in both FLD and MSD. MS analysis revealed that the molecular weight of M3 is 584, which is smaller by two atomic mass units than the parent compound CPT-11. A study on M3 (Fig. 1) and the enzyme responsible for this metabolite will be described elsewhere (Sai *et al.*, 2001). These results also suggested that parallel combined detection with MSD and FLD enabled more selective detection of minor metabolites and was useful for identification of this novel one rather than using FLD alone.

CONCLUSION

The validation results for the current analytical method indicated that this method was satisfactory for the determination of CPT-11 and its metabolites including minor CYP3A4-mediated products. Employing a parallel combination of FLD and MSD, the current method enabled a highly selective analysis for both major and minor CPT-11-metabolites among a variety of unknown compounds and without any interference by drugs possibly co-administered or their metabolites. Furthermore, with the current analytical system, a novel CPT-11 metabolite was identified in the human hepatic microsomes. Taken together, the current analytical method is expected to be useful for the studies on CPT-11 metabolisms in both *in vivo* and *in vitro* systems.

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