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A liquid chromatography/electrospray ionization mass spectrometry (LC–MS/MS) assay for the determination of irinotecan (CPT-11) and its two major metabolites in human liver microsomal incubations and human plasma samples

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ABSTRACT

A sensitive, rapid LC–MS/MS assay has been developed and validated for the simultaneous quantification of CPT-11 and its two principal metabolites, 7-ethyl-10-hydroxycamptothecin (SN-38), and 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxy-camptothecin (APC) in human liver microsomal fractions and plasma. The method was linear over the ranges of 1.56–100 ng/mL, 3.13–150 ng/mL, and 0.78–100 ng/mL for CPT-11, SN-38, and APC, respectively. The total run time was 7.0 min. This assay offers advantages in terms of expediency, recovery of analytes, and suitability for the analysis of CPT-11 and its metabolites in various biological fluids.

1. Introduction

Irinotecan (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin) is a camptothecin (CPT) derivative [1] that is used clinically for the treatment of colorectal cancer (Fig. 1). CPT-11 has also shown promising antitumor activity in other neoplastic disorders, including non-small cell lung carcinoma, cancer of the cervix, pancreatic and brain malignancies [2–4].

CPT-11 is a prodrug that requires the carboxylesterase-mediated biotransformation to the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) to exert its cytotoxic effects (Fig. 1); SN-38 inhibits the activity of topoisomerase I [5,6]. SN-38-mediated effects on rapidly dividing tissues, such as the intestinal mucosa and bone marrow, are responsible for the main dose-limiting toxicities in CPT-11 therapy such as diarrhea and myelosuppression [7,8].

The wide inter-patient variability observed in pharmacokinetic and pharmacodynamic properties of CPT-11 [8–10] has increased the difficulty of predicting CPT-11-induced toxicity. Indeed, the pharmacokinetic elimination of CPT-11 is mediated by drug metabolizing enzymes and transporters that exhibit inter-patient variability. Thus, CPT-11 is oxidized by cytochrome P450 3A (CYP3A) to the inactive metabolite 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxy-camptothecin (APC) (Fig. 1) [8]. APC has been shown to be a major plasma metabolite of CPT-11 in patients, and it is estimated that approximately 15% of a 125 mg/m^2 CPT-11 dose is excreted in this form [11,12]. The rate at which CPT-11 undergoes biotransformation to APC has been shown to indirectly influence the amount of the active metabolite SN-38 being formed [8,13]. These considerations underscore the potential utility of a rapid and convenient assay for the measurement of CPT-11 and its metabolites.

The plasma pharmacokinetics of CPT-11 in humans has now been addressed in several papers [14–18] but investigating CPT-11 biotransformation in an *in vitro* setting might provide valuable insight into the factors that influence the rate of formation of the two major plasma metabolites mentioned, SN-38 and APC. A flexible, quantitative assay that is applicable to different biological matrices is necessary for the timely and accurate transposition of *in vitro* findings to the clinical setting. A number of methods for the quantification of CPT-11 and its metabolites have been reported in the literature. The majority use analytical techniques such as high-performance liquid chromatography (HPLC) with either UV or fluorescence detection [16,19–25], but others employ HPLC coupled with mass spectrometry (MS) [11,13,26,27]. Even though MS methods generally have higher sensitivity for the analytes investigated, all of the methods to date, with the exception of Sai et al. [27] have described the detection of only one of the CPT-11 metabolites in either microsomal fractions or plasma. These assays require laborious sample preparation and are time consuming. Sai et al. [27] reported a method for analysis of CPT-11, SN-38, and APC in both human plasma and human liver microsomes; validation was undertaken in plasma. The present assay, however, offers advan-

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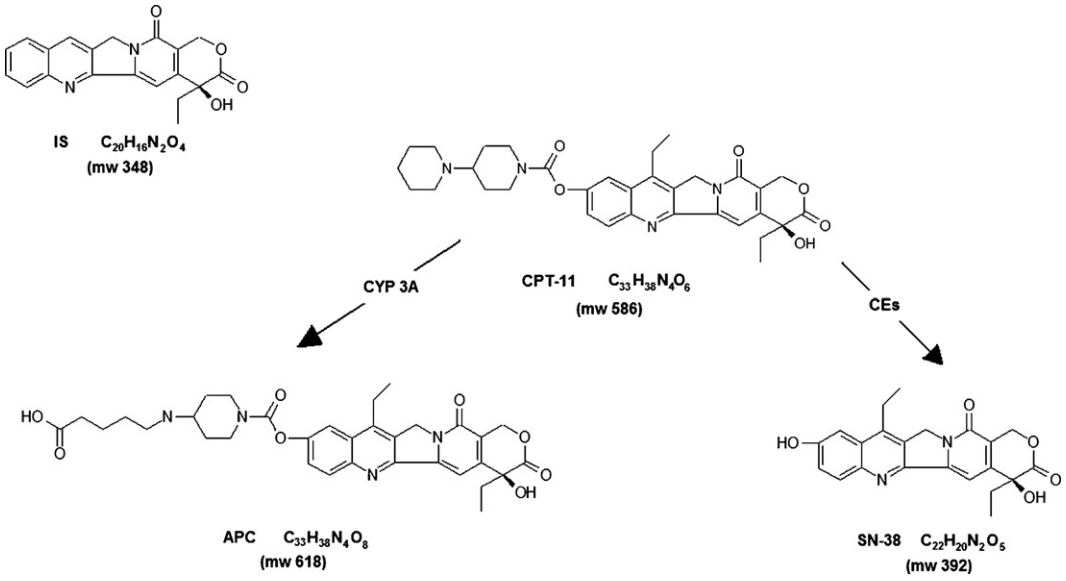

Fig. 1. Structures of CPT-11, SN-38, APC, and camptothecin (IS). Biotransformation of CPT-11 into SN-38 and APC is mediated by carboxylesterases (CEs) and cytochrome P450 3A (CYP 3A), respectively.

tages in terms of sensitivity and run time and was also validated in human liver microsomes.

The present paper describes the development and validation of a sensitive, specific, simple and rapid LC-tandem MS (LC–MS/MS) method for the simultaneous determination of CPT-11, SN-38 and APC in human liver microsomal preparations and plasma.

2. Experimental

2.1. Chemicals

CPT-11 was provided by Mayne Pharma (Mulgrave, VIC, Australia) as Irinotecan Injection Concentrate (25 mg/mL). SN-38 and APC were generously provided by Pfizer (West Ryde, NSW, Australia). Camptothecin (internal standard, IS) was purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Supergradient acetonitrile was purchased from Lab-Scan (Bangkok, Thailand). HPLC-grade water from a Milli-Q system (Millipore, North Ryde, NSW Australia) was used. All other chemicals were of analytical grade.

2.2. Preparation of human liver microsomal fractions

The *in vitro* experiments described in this study were performed using human liver microsomes obtained from healthy individuals as well as from patients suffering from hepatic cirrhosis and cholestatic liver disease. Approval for the use of these fractions was obtained from the University of Sydney Human Ethics Committee. The microsomal fractions were prepared by differential centrifugation as previously described by Murray and Murray [28] with minor modifications. Briefly, tissue was homogenized in 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 250 mM sucrose using a RZR 2100 electronic homogenizer (Heidolph, Germany) and centrifuged at 10,000 × *g* for 10 min (4 °C). The supernatant was centrifuged at 105,000 × *g* for 60 min (4 °C), followed by resuspension in buffer and resedimentation at 105,000 × *g* for 30 min (4 °C). The final microsomal pellets obtained were resuspended in 50 mM potassium phosphate buffer (pH 7.4) that contained 20% glycerol and 1 mM EDTA, snap frozen in liquid nitrogen and stored at −70 °C until required. Microsomal protein was determined by the method of Lowry et al. [29] using bovine serum albumin as standard.

2.3. Plasma samples

Blank human plasma from healthy volunteers was provided by SydPath (St Vincent's Hospital, Sydney, NSW, Australia).

2.4. LC–MS/MS instrument conditions

The analysis of the samples was performed on a Finnigan/Mat TSQ 7000 triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), operating in the positive electrospray ionization (ESI) mode. Chromatographic separation was carried out on a Hewlett-Packard HP 1090 LC controlled by the TSQ 7000 software. The analytes were separated using an Alltima C_{18} column (150 × 2.1 mm, 5 μm) (Alltech, Baulkham Hills, NSW, Australia) equipped with a 5 μm Alltima C_{18} 5 μ (7.5 × 2.1 mm) guard column cartridge (Alltech). A gradient mobile phase consisting of water (solvent A) and acetonitrile (solvent B) containing 0.25% formic acid was delivered at a flow rate of 0.3 mL/min. The composition of the mobile phase was switched from A:B 90:10 (v:v) to A:B 10:90 (v:v) over 1.0–5.0 min of the run before returning to A:B 90:10 (v:v) over 30 s. Retention times were 4.57 min for CPT-11, 4.92 min for SN-38, 4.53 min for APC, and 5.09 min for IS. The total run time was 7.0 min. The MS was operated in the selected reaction monitoring (SRM) mode. Nitrogen (HP, BOC, North Ryde, NSW, Australia) was used as the sheath gas at 70 psi; argon (UHP, BOC, North Ryde, NSW, Australia) was used as the collision gas at 2.2 mT. The MS parameters for analytes are listed in Table 1. The data acquisition was accomplished by Xcalibur 1.2 (Thermo Fisher Scientific).

2.5. Quality controls (QC) and calibration curves

Standard stock solutions of CPT-11 (0.1 mg/mL), SN-38 (0.1 mg/mL), APC (0.1 mg/mL) and IS (0.1 mg/mL) were prepared separately in methanol. Working solutions for calibration and controls were prepared by appropriate dilutions of separate stock solutions in phosphate buffer (pH 7.4).

Table 1
Ion source and analyte-dependent MS parameters.

Ion source				
Spray voltage (V)	4500			
Capillary temperature (°C)	275			
Sheath gas (psi)	70			
Polarity mode	Positive			
Collision gas (mT)	2.2			
Analyte dependent				
	CPT-11	SN-38	APC	IS
Precursor ion (m/z)	587	393	619	349
Product ion (m/z)	124	349	227	305
Collision energy	37.5	30	25	30

2.5.1. Microsomal fractions

QC samples for microsomal fraction assays were prepared in triplicate at concentrations of: 1.56 (lower limit of quantification (LLOQ)), 5.00 ng/mL (low), 25.00 ng/mL (medium), and 100.00 ng/mL (high) for CPT-11; 3.13 ng/mL (LLOQ), 10.00 ng/mL (low), 75.00 ng/mL (medium), and 150.00 ng/mL (high) for SN-38; 0.78 ng/mL (LLOQ), 2.50 ng/mL (low), 25.00 ng/mL (medium), and 100.00 ng/mL (high) for APC. Working solutions of the analytes (50 μL) and IS (50 μL, working stock concentration 62.5 ng/mL) were added to drug-free microsomal incubation extracts (2.5 mL). The mixture was subsequently evaporated to dryness at 40 °C under a stream of nitrogen and the residue was resuspended in 10 μL of methanol and 190 μL of mobile phase.

Calibration curves were constructed from a blank sample (microsomal fractions from which the IS was excluded), a zero sample (microsomal fractions containing the IS) and seven non-zero samples spanning the total range of concentrations used, including the LLOQ. The following concentrations were obtained in triplicate using the procedure described for QC sample preparation: CPT-11 – 1.56, 5.00, 10.00, 25.00, 50.00, 75.00, and 100.00 ng/mL; SN-38 – 3.13, 10.00, 25.00, 50.00, 75.00, 100.00, and 150.00 ng/mL; APC – 0.78, 2.50, 5.00, 10.00, 25.00, 50.00, and 100.00 ng/mL; the final IS concentration was 15.6 ng/mL.

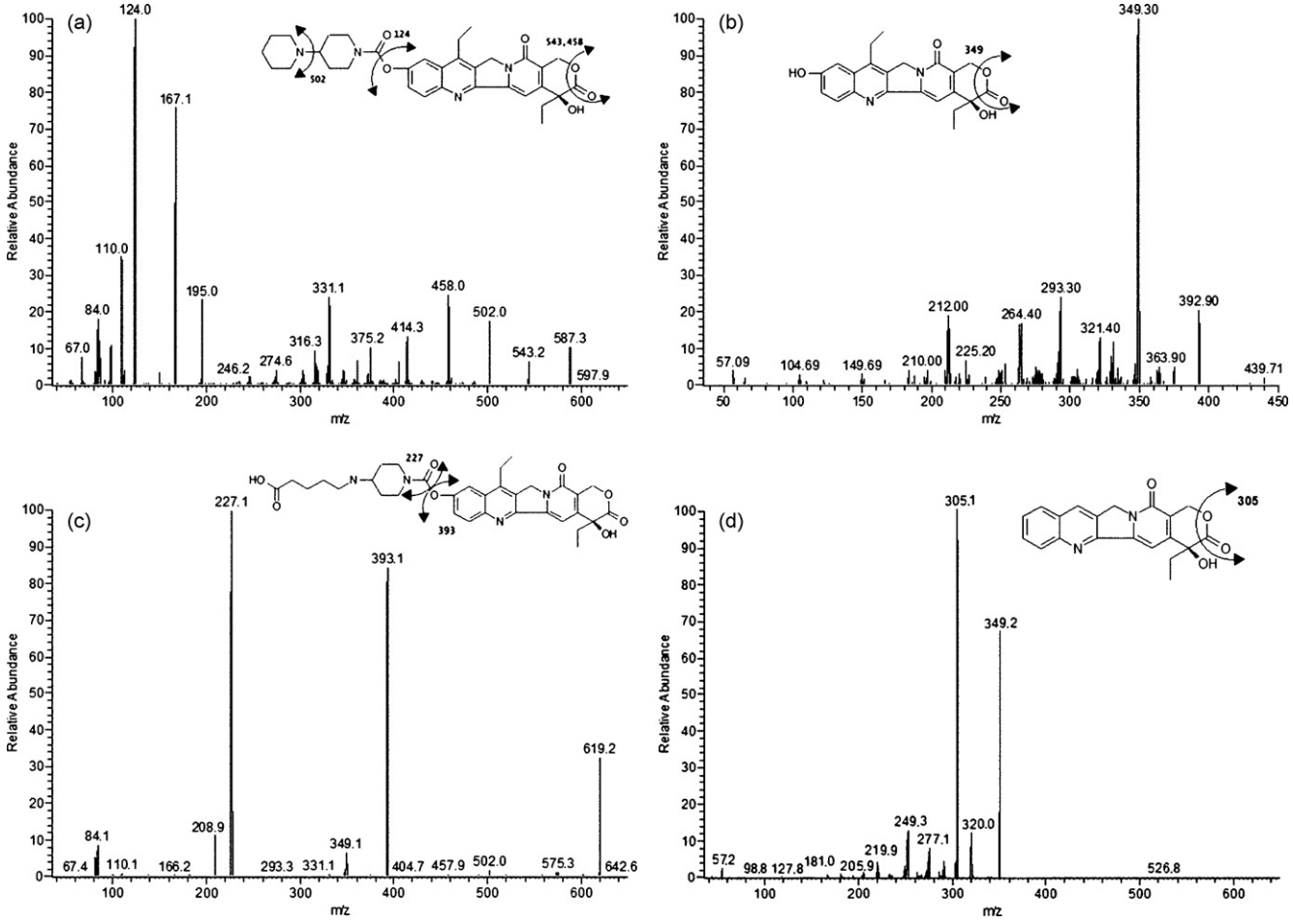

Fig. 2. ESI product ion mass spectra for the precursor ion, and proposed fragmentation patterns of (a) CPT-11, (b) SN-38, (c) APC, and (d) IS.

2.5.2. Plasma samples

QC samples for human plasma assays were prepared in triplicate at concentrations of: 1.56 ng/mL (LLOQ), 2.50 ng/mL (low), 12.50 ng/mL (medium), and 25.00 ng/mL (high) for CPT-11; 3.13 ng/mL (LLOQ), 5.00 ng/mL (low), 25.00 ng/mL (medium), and 150.00 ng/mL (high) for SN-38; 0.78 ng/mL (LLOQ), 1.30 ng/mL (low), 7.50 ng/mL (medium), and 25.00 ng/mL (high) for APC. Working solutions of the analytes (50 μL) and IS (50 μL, working stock concentration 62.5 ng/mL) were added to drug-free plasma sample extracts (2.5 mL). The mixture was subsequently evaporated to dryness at 40 °C under a stream of nitrogen and the residue was resuspended in 10 μL of methanol and 190 μL of mobile phase.

Calibration curves were constructed from a blank sample (plasma sample from which the IS was excluded), a zero sample (plasma sample containing the IS) and seven non-zero samples spanning the total range of concentrations used, including the

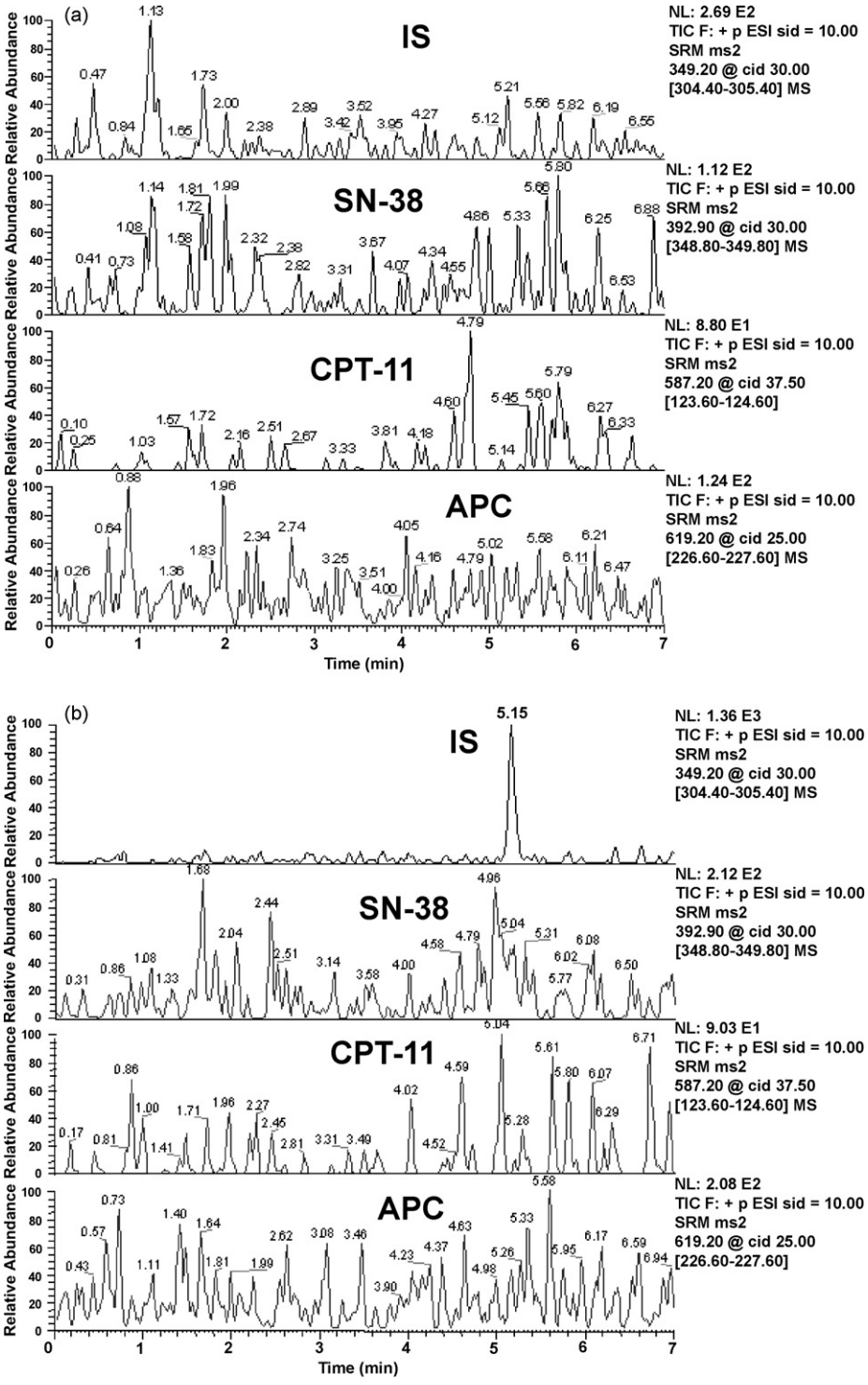

Fig. 3. Representative chromatograms obtained from (a) an extracted blank microsomal fraction, (b) an extracted blank human microsomal fraction spiked with IS, and (c) an extracted human microsomal sample containing low concentrations of CPT-11 (5.00 ng/mL), SN-38 (10.00 ng/mL) and APC (2.50 ng/mL).

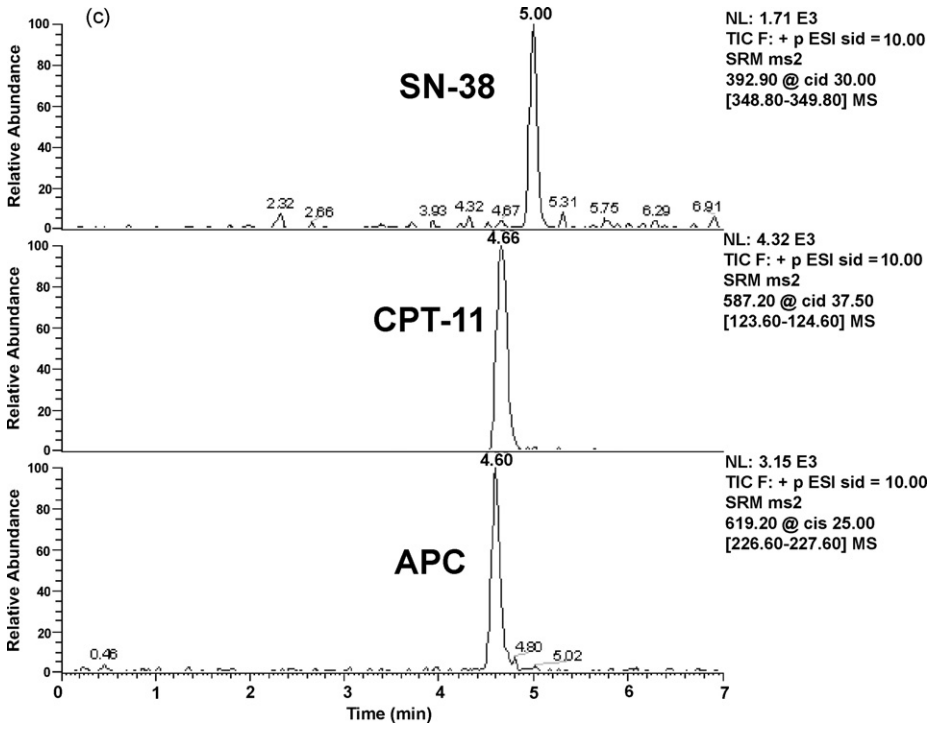

Fig. 3. (*Continued*)

LLOQ. The following concentrations were obtained in triplicate using the procedure described for QC sample preparation: CPT-11 – 1.56, 2.50, 3.80, 7.50, 12.50, 18.80, and 25.00 ng/mL; SN-38 – 3.13, 5.00, 7.50, 12.50, 18.80, 25.00, and 150.00 ng/mL; APC – 0.78, 1.30, 2.50, 3.80, 7.50, 12.50, and 25.00 ng/mL; the final IS concentration was 15.6 ng/mL.

The calibration curves were generated using the analyte to IS peak area ratios. The acceptance criteria for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within ±15% deviation from the nominal value, except at the LLOQ, for which the maximum acceptable deviation was set at ±20%.

2.6. Sample preparation

Solid phase extraction (SPE) was used to process the samples prior to LC–MS/MS analysis. Working solutions (50 μL) of each of the analytes were added to human liver microsomal preparations in phosphate buffer (0.2 mg/mL) to obtain a total incubation volume of 500 μL. For preparation of plasma samples, working solutions (50 μL) of each of the analytes in methanol were added to microcentrifuge tubes and reduced to dryness under nitrogen at 40 °C. The analytes were then reconstituted in 500 μL of plasma.

The samples were spiked with 50 μL of 62.5 ng/mL IS (final concentration 15.6 ng/mL), before addition of 950 μL of acetonitrile:methanol 50:50 (v:v). Samples were vortex-mixed for 1 min and centrifuged at 15 000 × g for 10 min (4 °C) to sediment the protein.

The supernatant (1 mL) was reduced to dryness under nitrogen at 40 °C. The samples were then reconstituted in 1.5 mL of 1% formic acid and transferred to SPE Bond Elut Plexa cartridges (polymer beads, 1 mL, 30 mg) (Varian, Melbourne, VIC, Australia) which were pre-conditioned by washing with 2.5 mL of methanol followed by 2.5 mL of water. An ASPEC XL4 automated SPE system (Gilson, Middleton, WI, USA) was used to house the cartridges. Supernatant fractions were run through the cartridges at a flow rate of 0.5 mL/min. The cartridges were then washed with 2.5 mL of 5% methanol in water, and the analytes were eluted with 2.5 mL of methanol at a flow rate of 0.5 mL/min. The solvent was evaporated to dryness under nitrogen at 40 °C, and the residue was reconstituted with 10 μL of methanol and 190 μL of mobile phase. The samples were then transferred to an autosampler vial and a total volume of 20 μL was injected into the LC–MS system.

2.7. Validation

The method was validated for ion suppression, selectivity, sensitivity, linearity, precision, accuracy, and recovery. Ion suppression from the extraction procedure was determined by comparison of the peak areas of pure analytes in mobile phase with the peak areas of analytes which were added to human liver microsomal fractions (n = 6) and plasma samples (n = 6) following SPE. Investigation of selectivity was performed by analyzing blank microsomal fractions and plasma samples from different sources to test for interference at the retention times of analytes and IS. The intra- and inter-batch precision and accuracy were determined by replicate analysis of QC samples and samples at LLOQ. The acceptance criteria for within- and between-batch precision were 20% or less of the coefficient of variation (CV) for LLOQ and 15% or less of the CV for the other concentrations, and for accuracy were 80–120% of the nominal value at the LLOQ and 85–115% of the nominal value at the other concentrations. Recovery of CPT-11, SN-38, and APC from the extraction procedure was determined by comparisons of the peak areas of each of the analytes in spiked human liver microsomal fractions and plasma samples (five each of low, medium, and high QCs) with the peak areas of CPT-11, SN-38, APC, and IS in samples prepared by spiking extracted drug-free human liver microsomal fractions and plasma samples with the same amount of analytes at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing

the mean peak areas of extracted QC samples ($n = 5$) to mean peak areas of IS in samples prepared by spiking extracted drug-free microsomal fractions and plasma samples with the same amounts of IS at the step immediately prior to chromatography.

A number of studies have determined the stability of CPT-11 and its metabolites in methanolic solutions and in biological matrices. Stability of the analytes was tested at room temperature and at 37 °C for 20 h, at 4 °C for 0, 5, 22, 28, and 41 days, at −80 °C for 2 months, and over five freeze-thaw cycles [20,27,30,31].

2.8. Microsomal incubations conditions

Time, protein, and substrate linearity tests were performed to optimize the incubation conditions. Microsomal fractions from five healthy individuals and five individuals with chronic liver disease (final protein concentration: 0.2 mg/mL) were incubated with CPT-11 (final concentration 5 μM) in phosphate buffer (pH 7.4; reaction volume 500 μL) at 37 °C for 5 min. Reactions were initiated by the addition of β-NADPH (final concentration, 1 mM) and terminated after 20 min by the addition of 950 μL of cold acetonitrile:methanol (1:1, v:v). The IS (final concentration, 1.56 ng/mL)

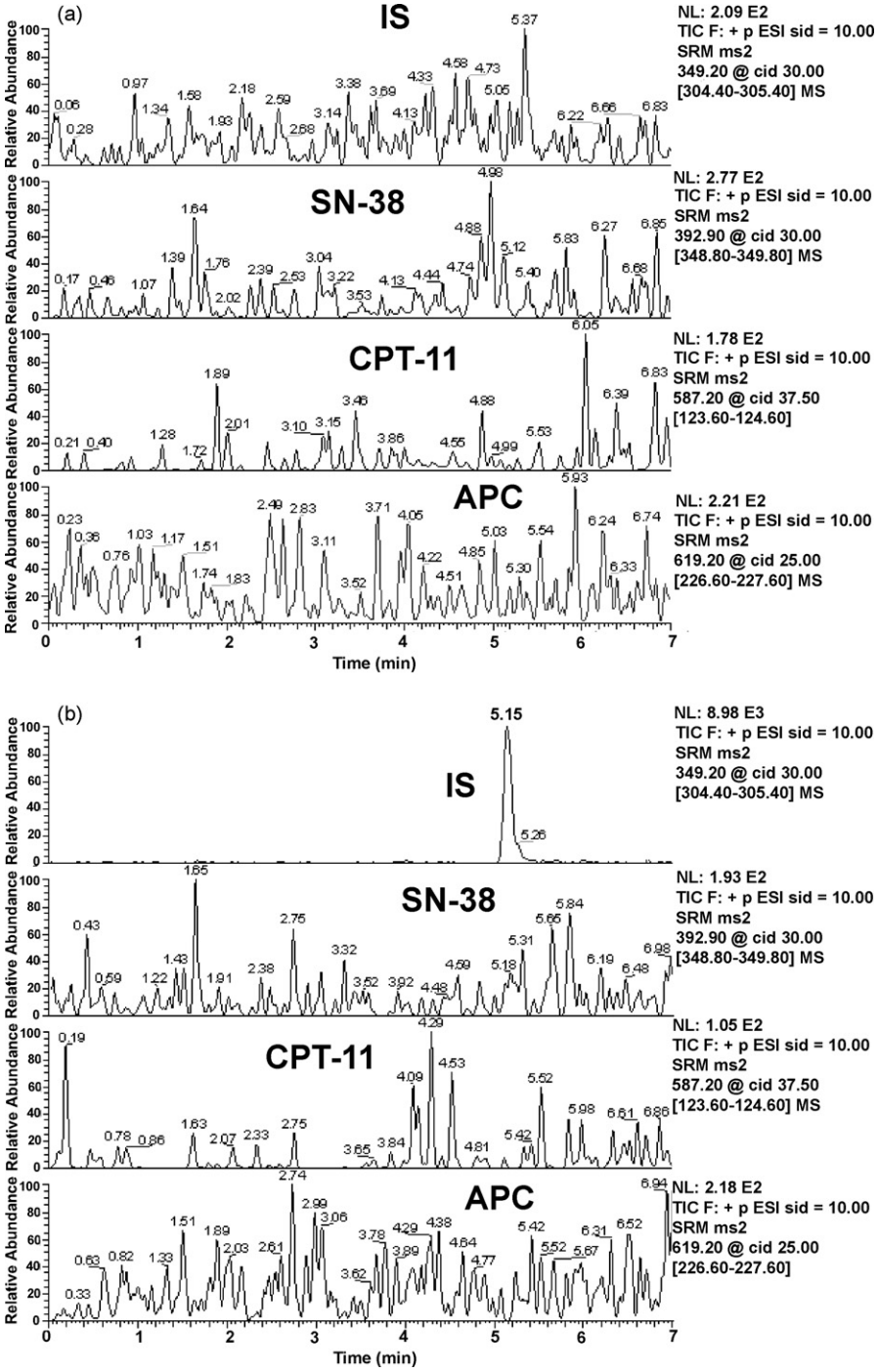

Fig. 4. Representative chromatograms obtained from (a) an extracted blank plasma sample, (b) an extracted blank plasma sample spiked with IS, and (c) an extracted plasma sample containing low concentrations of CPT-11 (2.50 ng/mL), SN-38 (5.00 ng/mL) and APC (1.30 ng/mL).

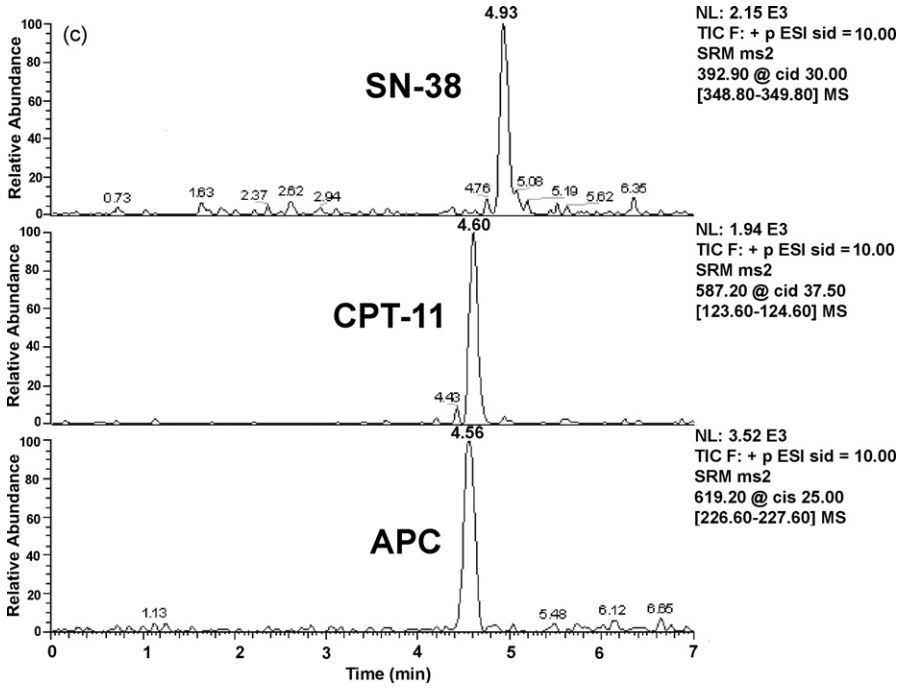

Fig. 4. (*Continued*)

Table 2
Linearity and intercept of CPT-11, SN-38, and APC calibration curves ($n = 7$).

	Microsomal fractions			Plasma samples		
	Linear range (ng/mL)	Correlation coefficient	Intercept mean (±SD)	Linear range (ng/mL)	Correlation coefficient	Intercept mean (±SD)
CPT-11	1.56–100	0.9909	0.0892 (±0.0044)	1.56–25	0.9998	0.1815 (±0.0160)
SN-38	3.13–150	0.9905	0.0467 (±0.0032)	3.13–150	1.0000	0.0858 (±0.0076)
APC	0.78–100	0.9986	0.0188 (±0.0132)	0.78–25	0.9996	0.2718 (±0.0118)

was then added to all samples before SPE extraction and analysis as described.

3. Results and discussion

3.1. Mass spectrometry

A sensitive, rapid LC–MS/MS assay has been developed and validated for the simultaneous quantification of CPT-11 and its two principal metabolites, SN-38 and APC in human liver microsomal fractions and human plasma samples. Detection parameters, chromatography and sample extraction were optimized during development of the method.

Full scan positive ion spectra for CPT-11, SN-38, APC and IS gave protonated molecules ($[M+H]^+$) of m/z 587.20, m/z 392.90, m/z 619.20, and m/z 349.20, respectively. MS/MS fragmentation of these ions was monitored using instrument settings that optimized intensity to one major product ion for each component (Table 1);

Table 3
Intra-batch precision and accuracy ($n = 5$) of CPT-11, SN-38, APC, and IS.

	Microsomal fractions				Plasma samples			
	Concentration		Precision (%)	Accuracy (%)	Concentration		Precision (%)	Accuracy (%)
	Added (ng/mL)	Found (mean ± SD) (ng/mL)			Added (ng/mL)	Found (mean ± SD) (ng/mL)		
CPT-11	1.56	1.76 ± 0.12	6.86	112.82	1.56	1.63 ± 0.29	17.60	104.49
	5.00	5.68 ± 0.18	3.18	113.60	2.50	2.31 ± 0.24	10.57	92.40
	25.00	24.62 ± 3.66	14.87	98.48	12.50	13.47 ± 1.67	12.39	107.76
	100.00	100.12 ± 9.21	9.19	100.12	25.00	23.42 ± 1.90	8.09	93.68
SN-38	3.13	2.81 ± 0.29	10.30	89.78	3.13	3.69 ± 0.23	6.10	117.89
	10.00	10.02 ± 0.91	9.07	100.20	5.00	5.48 ± 0.33	6.06	109.60
	75.00	73.61 ± 7.68	10.43	98.15	25.00	25.06 ± 0.79	3.12	100.24
	150.00	157.68 ± 6.51	4.13	105.12	150.00	147.41 ± 10.23	6.94	98.27
APC	0.78	0.69 ± 0.11	15.68	88.46	0.78	0.85 ± 0.07	8.09	108.97
	2.50	2.39 ± 0.25	10.62	95.60	1.30	1.25 ± 0.52	9.76	96.15
	25.00	25.53 ± 1.57	6.15	102.12	7.50	7.72 ± 0.65	8.44	102.87
	100.00	96.02 ± 8.59	8.94	96.02	25.00	22.58 ± 1.00	4.44	90.32

Table 4
Inter-batch precision and accuracy ($n = 3$) of CPT-11, SN-38, APC, and IS.

	Microsomal fractions				Plasma samples			
	Concentration		Precision (%)	Accuracy (%)	Concentration		Precision (%)	Accuracy (%)
	Added (ng/mL)	Found (mean ± SD) (ng/mL)			Added (ng/mL)	Found (mean ± SD) (ng/mL)		
CPT-11	1.56	1.49 ± 0.12	15.09	95.51	1.56	1.55 ± 0.18	11.29	99.35
	5.00	5.64 ± 0.73	12.16	112.80	2.50	2.45 ± 0.31	12.63	98.00
	25.00	24.65 ± 1.62	6.55	98.60	12.50	13.12 ± 1.28	9.78	104.96
	100.00	100.68 ± 3.81	3.78	100.68	25.00	23.60 ± 2.56	10.83	94.40
SN-38	3.31	3.49 ± 0.15	4.17	105.44	3.13	3.28 ± 0.39	11.83	104.79
	10.00	10.74 ± 1.22	11.35	107.40	5.00	5.62 ± 0.49	8.69	112.40
	75.00	82.62 ± 1.27	4.78	110.16	25.00	24.70 ± 2.57	10.41	98.80
	150.00	164.17 ± 3.36	2.05	109.45	150.00	150.47 ± 12.29	8.17	100.31
APC	0.78	0.76 ± 0.13	17.70	97.44	0.78	0.80 ± 0.13	16.69	102.56
	2.50	2.31 ± 0.15	6.73	92.40	1.30	1.34 ± 0.14	10.36	103.08
	25.00	24.66 ± 1.61	6.55	98.64	7.50	7.41 ± 0.65	8.79	98.80
	100.00	100.03 ± 10.80	10.80	100.03	25.00	23.7 ± 1.00	4.23	94.80

the predominant and specific fragment of each compound was selected and used as the product ion to be monitored (Table 1). Fig. 2 shows the product ion spectra and proposed fragmentation patterns of the analytes and IS.

3.2. Method development

The chromatographic conditions were optimized through several trials to achieve good sensitivity and peak symmetry for the analytes and IS, as well as short run time.

SPE was used for the sample preparation. A number of different options were evaluated to optimize all steps of sample extraction. Precipitation of sample protein and addition of 1.5 mL of 1% formic acid prior to extraction improved the retention of material on the solid phase and the elution of analytes, as well as improving LC–MS/MS peak shape. The acidic conditions used during SPE also prevented hydrolysis of the lactone form of the analytes to the corresponding, open-ring carboxylate forms. Because these two chemical configurations have different chromatographic profiles, this enabled a more rapid determination of analyte concentration and improved analyte stability. A range of aqueous methanol solutions was used to wash the cartridges following the loading of analyte solutions: 5% methanol in water optimized analyte recovery and minimized background noise in the MS spectra. Finally it was found that none of the combinations of solvents tested further improved the elution of analytes obtained with methanol alone.

Although the use of isotopically labeled internal standards for all analytes was preferred, these were not commercially available. Instead camptothecin was selected as the IS because this compound is structurally similar to the analytes and is readily available.

3.3. Method performance and validation

3.3.1. Ion suppression

To investigate ion suppression the responses of calibrators injected into the mobile phase were compared with responses to the same amount of CPT-11, SN-38, APC, and IS added to extracted microsomal fractions and plasma samples. To ensure that slight differences in matrix did not influence ion suppression six different samples of both matrices were used. A small decrease in signal was observed for all analytes and IS, but because the reduction in signal for the IS and the other analytes coincided, the observed ion suppression was corrected when concentrations were calculated. When assessed for ion suppression the signal decreased by less than 20% when analytes were spiked into extracted microsomal fractions, and by less than 15% when spiked into extracted plasma samples.

3.3.2. Selectivity

The selectivity of the method was examined by analyzing ($n = 5$) blank microsomal fraction and ($n = 5$) plasma sample extracts (Figs. 3a and 4a) and extracts spiked only with the IS (Figs. 3b and 4b). As shown in Figs. 3a and 4a, direct interference in the blank microsomal and plasma chromatograms by endogenous substances at the retention time of the analytes was minimal. Similarly, Figs. 3b and 4b show the absence of direct interference from the IS to the SRM channel of the analytes. Figs. 3c and 4c depict representative ion chromatograms at the LLOQ for CPT-11, SN-38 and APC. Satisfactory sensitivity was observed for a 20 μL injection volume: the LLOQ corresponds to ca. 31.25, 62.50, and 15.63 pg of CPT-11, SN-38, and APC on-column, respectively.

3.3.3. Linearity

The calibration curves were linear (not shown). Peak area ratios of calibration standards to IS in microsomal extracts were proportional to the concentration of the analytes over the concentration ranges used (Table 2).

3.3.4. Sensitivity (LLOQ), precision, and accuracy

The intra- and inter-batch LLOQ, precision, and accuracy for CPT-11, SN-38, and APC in microsomal fractions and plasma samples are shown in Tables 3 and 4.

3.3.5. Recovery

Five replicates at low, medium, and high QC concentrations for CPT-11, SN-38, and APC were prepared for recovery determination; the mean recoveries from microsomal fractions were 65.77%, 80.44%, and 67.43%, respectively. Recovery of the IS was 73.58% at the concentration used in the assay (15.6 ng/mL). Although recovery of CPT-11 and APC may appear suboptimal, these are comparable to previously published SPE extraction efficiencies [16,26], and, most importantly, were found to be consistent and reproducible. In addition strong correlations were found between nominal concentration of analytes and the ratio of mean recovered analyte peak areas to mean recovered IS peak areas; the relevant r^2 values for these correlations were as follows: CPT-11 – $r^2 = 0.999$, SN-38 – $r^2 = 1.000$, and APC – $r^2 = 1.000$. Such correlations would enable the accurate determination of analyte concentrations in unknown samples. The mean recoveries from plasma samples were 94.07%, 90.47%, and 85.63%, respectively. Recovery

of the IS was 75.86% at the concentration used in the method (15.6 ng/mL).

3.3.6. Stability

A number of studies have determined the stability of CPT-11 and its metabolites in methanolic solutions and in biological matrices. The analytes were found to be stable at 4 °C for at least 41 days when stored in methanol [30]. CPT-11 and its metabolites in plasma have been shown to be stable for up to 20 h at room temperature and for up to at least 8 weeks when stored at −80 °C. Analytes in plasma were also found to be stable after five freeze-thaw cycles. Data from stability studies indicated that the lactone forms of CPT-11 and SN-38 were unstable during prolonged storage at room temperature or at 37 °C [20,27,31]. This instability requires rapid freezing of clinical samples after blood collection to prevent significant degradation of lactone into carboxylate forms. The determination of total CPT-11, SN-38, and APC concentrations by sample acidification overrides this requirement.

3.4. Assay application

The validated method was applied to investigate CPT-11 biotransformation in microsomal fractions from healthy individuals and patients with liver disease. The formation of SN-38 and APC ranged from 41.5 to 150.3 nmol/min/mg of protein and from 1.4 to 7.5 nmol/min/mg of protein, respectively. Thus, the present assay is suitable for the rapid determination of CPT-11 and its two major metabolites in microsomal fractions and other biological matrices.

4. Conclusion

A simple, rapid LC–MS/MS method for the determination of CPT-11, SN-38, and APC in both human liver microsomal fractions and plasma has been developed. The current method exhibits acceptable precision and recovery of analytes, acceptable sensitivity, excellent linearity and short run time.

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