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Short communication

Simultaneous determination of irinotecan (CPT-11) and SN-38 in tissue culture media and cancer cells by high performance liquid chromatography: Application to cellular metabolism and accumulation studies

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Abstract

A simple and sensitive HPLC method was developed to simultaneously determine CPT-11 and its major metabolite SN-38 in culture media and cell lysates. Camptothecin (CPT) was used as internal standard (I.S.). Compounds were eluted with acetonitrile–50 mM disodium hydrogen phosphate buffer containing 10 mM sodium 1-heptane-sulfonate, with the pH adjusted to 3.0 using 85% (w/v) orthophosphoric acid (27/73, v/v) by a Hyperclon ODS (C18) column (200 mm × 4.6 mm i.d.), with detection at excitation and emission wavelengths of 380 and 540 nm, respectively. The average extraction efficiencies were 96.9–108.3% for CPT-11 in culture media and 94.3–107.2% for CPT-11 in cell lysates; and 87.7–106.8% for SN-38 in culture media and 90.1–105.6% for SN-38 in cell lysates. Within- and between-day precision and accuracy varied from 0.1 to 10.3%. The limit of quantitation (precision and accuracy <20%) was 5.0 and 2.0 ng/ml for CPT-11 and 1.0 and 0.5 ng/ml for SN-38 in culture media and cell lysates, respectively. This method was successfully applied to quantitate the cellular accumulation and metabolism of CPT-11 and SN-38 in H4-II-E, a rat hepatoma cell line.
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Keywords: CPT-11; SN-38; Metabolism; Accumulation; HPLC

1. Introduction

Irinotecan hydrochloride (CPT-11, Fig. 1), a semisynthetic derivative of camptothecin (CPT), has been widely used for the treatment of advanced colorectal cancer and many of other solid tumours [1]. CPT-11 as a prodrug is hydrolyzed by carboxylesterases to form the active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin). SN-38 is further converted to its glucuronide (SN-38G) by uridine diphosphate glucuronosyltransferases 1A and 1A9, which is then eliminated by biliary excretion [2]. Both CPT-11 and SN-38 have two forms co-existing: the active lactone form and the inactive carboxylate form. The lactone form of CPT-11 and SN-38 has a closed α-hydroxy-δ-lactone ring, which can be hydrolyzed to form the open-ring hydroxyl acid (carboxylate form).

Several high performance liquid chromatographic (HPLC) methods have been developed for the simultaneous determination of CPT-11 and SN-38 in buffered solutions and biological fluids [3–9]. Boyd et al. [9] reported the HPLC methods with fluorescence detection for simultaneous determination of lactone and hydroxy acid (carboxylate) forms of CPT and SN-38 in tissue culture media and cancer cells, but no bio-analytical assay has been validated for determination of total closed form of CPT-11 and SN-38 in culture media and cell lysates. In order to study the cellular pharmacology of CPT-11 analogs, we described a simple and sensitive isocratic HPLC method for the simultaneous determination of CPT-11 and SN-38 in tissue culture media and cancer cells with CPT as internal standard (I.S.). The plasma concentrations of CPT-11 and SN-38 in lactone form are correlated with the levels of total closed form of these two compounds [3,4,6,10,11]. Thus, most clinical and preclinical studies have determined the total concentrations of two forms of

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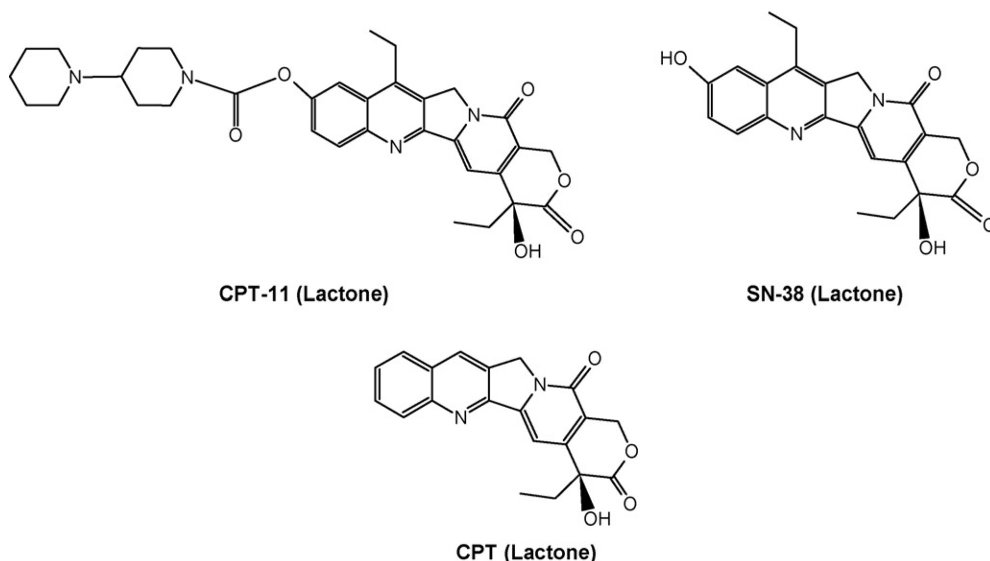

Fig. 1. Chemical structures of CPT-11, SN-38 and CPT.

CPT-11 and SN-38 by transferring carboxylate forms to lactone forms through acidification of the sample [11–13] or by transferring lactone forms to carboxylate forms through basification of the sample [10]. The simultaneous measurement of CPT-11 and SN-38 in both lactone and carboxylate forms requires more complicated separation procedure and longer chromatographic running times. Sometimes it needs several different HPLC conditions for each sample to determine the lactone form and total form separately. Therefore, the measurement of total closed form of CPT-11 and SN-38 is a suitable marker in the studies of cellular pharmacology of camptothecins.

2. Experimental

2.1. Chemicals and reagents

CPT analogues including CPT-11 and SN-38 (all compounds with a purity >99.0%) were purchased from SinoChem Ningbo Import and Export Co. (Ningbo, China). CPT (with a purity of 95.0%), the ion-pairing reagent sodium 1-heptane-sulfonate, lyophilized type IX-A β-glucuronidase (from *Escherichia coli*, with activity of 1,724,400 U/g solid form), Dulbecco's modified Eagle's medium (DMEM) were all purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Hyclone Lab Inc. (Logan, UT, USA). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore, Bedford, MA). All other reagents were of analytical grade available commercially.

2.2. Apparatus and chromatographic conditions

Chromatographic analyses were performed using a Shimadzu HPLC system (Nakagyo-ku, Kyoto, Japan) consisted of a LC-10AT$_{VP}$ pump, a DGU-14A on-line solvent degasser, a RF-10A XL fluorescence detector and a SIL-10AD$_{VP}$ autoinjector. Shimadzu Class-LC10 Workstation was used for system control and data were monitored and analyzed using Shimadzu CLASS VP software. The analytical column used was packed with Hyperclon ODS material (200 mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA) and protected by a Phenomenex C18 guard column (Torrance, CA). The mobile phase comprised of acetonitrile–50 mM disodium hydrogen phosphate buffer containing 10 mM sodium 1-heptane-sulfonate, with the pH adjusted to 3.0 with 85% (w/v) orthophosphoric acid (27/73, v/v). The mobile phase was delivered at a flow-rate of 1.0 ml/min, and the column effluent was monitored at 540 nm with an excitation wavelength of 380 nm. At an emission wavelength of 540 nm and an excitation wavelength of 380 nm, both CPT-11 and SN-38 in lactone form produced almost maximum response.

2.3. Cell culture

The H4-II-E, a rat hepatoma cancer cell line, was obtained from the American Type Culture Collection (Manassas, VA) and cultured in complete DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were grown in an atmosphere of 5% CO_2/95% air at 37 °C and given fresh medium every 3 or 4 days. Experiments were performed on cells within 10 passages. Viable cells were counted using the trypan blue exclusion method.

2.4. Standard solution and sample preparation

Stock solutions of CPT-11, SN-38 and CPT (I.S.) were prepared in dimethyl sulfoxide (DMSO) at the concentration of 1 mg/ml and working solutions were obtained by serial dilutions with ice-cold 0.01 M HCl:methanol mixture (1:1, v/v). All stock and working solutions were stored at −20 °C under dark condition and appeared to remain stable for more than 1 month.

Media standards were prepared by adding equal volume (100 μl) of the appropriate working solutions of CPT-11

and SN-38 as well as 100 μl CPT (final 0.1 μg) to a tube containing 100 μl fresh blank media. After vortex-mixing and centrifugation at 3000 × *g* for 10 min, the supernatant (100 μl) was injected onto the HPLC. Cell monolayers were washed five times with ice-cold phosphate-buffered saline (PBS), and mechanically detached from the flasks by scraping with the addition of 5 ml ice-cold PBS. After gentle vortex, cell pellets were harvested by centrifugation at 2000 × *g* for 5 min. Cell lysates standards were prepared by suspension of each cell pellet in equal volume (50 μl) of CPT-11 and SN-38 working solutions, I.S. CPT and extraction solution (0.01 M HCl:methanol, 1:1, v/v). After vortex-mixing, the cellular extracts were sonicated and centrifuged at 15,000 × *g* for 15 min, and the supernatant (100 μl) was then injected onto HPLC for analysis. Both media and cellular standards were prepared freshly.

Calibration curves were generated in the concentration ranges of 5–42,000 ng/ml in tissue culture media and 2–42,000 ng/ml in cell lysates for CPT-11; and 1–1500 ng/ml in tissue culture media and 0.5–1500 ng/ml cell lysates for SN-38. They were constructed by plotting the peak area ratio of the analytes over IS versus the concentrations spiked using unweighted least-squares linear-regression analysis. Because of the wide range of the concentrations studied, we constructed the calibration curves at two concentration ranges (5–42,000 ng/ml in tissue culture media and 2–42,000 ng/ml in cell lysates for CPT-11; and 1–1500 ng/ml in tissue culture media and 0.5–1500 ng/ml cell lysates for SN-38) in culture media and cell lysates, respectively.

2.5. Method validation

All validation runs were performed on 3 consecutive days and all samples used for validation were prepared as standard samples. Six different concentrations of CPT-11 and SN-38 were investigated for recovery, whereas CPT (the internal standard) was measured at the concentration used in sample preparation. The recovery was determined by comparing the analytical results for extracted samples with unextracted standards that represent 100% recovery.

Quality control samples containing each analyte (CPT-11: 2, 10, 100, 1000, 10,000 and 42,000 ng/ml in cell lysates, and 5, 10, 100, 1000, 10,000 and 42,000 ng/ml in culture media; SN-38: 0.5, 5, 20, 100, 500, 1500 and 2500 ng/ml in cell lysates, and 0.5, 5, 20, 100, 500, 1500 and 2500 ng/ml in culture media) were prepared from weighing independent of those used for preparing calibration curves. Within- and between-day precision and the mean accuracy were determined by analysis of both CPT-11 and SN-38 in triplicate on a single day and on 3 consecutive days, respectively. During each analytical run, QC samples were included and processed as the calibration and unknown samples. The limit of quantification (LOQ) was defined as the lowest drug concentration that could be determined with acceptable precision [i.e. coefficient of variation (CV) $\leq$20%] and accuracy (i.e. recovery of 100 ± 20%). The limit of detection was defined as the amount that could be detected with a signal-to-noise ratio of 3.

2.6. Cellular metabolism and accumulation studies

H4-II-E cells were grown in BD Falcon™ cell culture dishes (BD Biosciences, San Jose, CA) for metabolism and intracellular accumulation studies. Different concentrations of CPT-11 (0.5–50 μM, i.e. 174.2–17420.0 ng/ml) and SN-38 (0.1–5 μM, i.e. 39.24–1962.00 ng/ml) in DMEM medium were added to confluent cell cultures. An aliquot of the culture medium (0.1 ml) was collected from each dish over 120 min and concentrations of CPT-11 and SN-38 in culture media were determined as described above for metabolism study. The production of SN-38G was determined with the conversion of SN-38G to SN-38 by incubation with β-glucuronidase. For SN-38G analysis, the β-glucuronidase was dissolved in 0.1 M sodium phosphate buffer (pH 6.4) to obtain a concentration of 20,000 U/ml. An aliquot of culture media (50 μl) was incubated in water bath with 50 μl of the solution of β-glucuronidase (1000 U) for 2 h at 37 °C. Then the samples were processed by the same procedures as for CPT-11 and SN-38, except that the volumes of all the added solutions were doubled.

Intracellular accumulation of CPT-11 (0.5–50 μM, i.e. 174.2–17420.0 ng/ml) or SN-38 (0.1–5 μM, i.e. 39.24–1962.00 ng/ml) was also tested by determination of CPT-11 or SN-38 concentration in cell lysates over 120 min using the method described above. Viable cells were monitored using the trypan blue exclusion method. The productions of SN-38 and SN-38G and the intracellular accumulation of CPT-11 and SN-38 were expressed as pmol/min/10^6 cells.

3. Results and discussion

Representative chromatograms for CPT-11 and SN-38 in tissue culture media and cell lysates are shown in Figs. 2 and 3. The retention times for CPT-11, SN-38 and CPT were 10.04 ± 1.06, 6.15 ± 0.06 and 7.80 ± 0.07 min (n = 40), respectively. The peaks for SN-38 and CPT were slightly skewed to the right with asymmetry coefficients were between 1.03 and 1.10. The peak of CPT-11 was remarkably skewed to the right under the chromatographic conditions, probably due to the effect of low pH and other unknown factors. The asymmetry coefficients were between 1.15 and 1.31. The skewing of CPT-11 peak did not affect the quantitativity in peak area. We evaluated peak skew using the asymmetry coefficient $A_s = b/a$, where b is the distance after the peak maximum and a is the distance before the peak maximum, both a and b being measured at 10% of the total peak height.

These methods employed a simple protein precipitation step, with 87.7–106.8% recovery for both compounds at tested concentrations (Table 1). The average extraction efficiencies were 96.9–108.3% for CPT-11 in culture media and 94.3–107.2% for CPT-11 in cell lysates; and 87.7–106.8% for SN-38 in culture media and 90.1–105.6% for SN-38 in cell lysates. Sample preparation conditions were optimized to one step by the means of incorporation of I.S. (CPT) to samples in acidified methanol solution. In the HPLC method using a solid-phase extraction approach utilizing C2-bonded silica particles (100 mg, 1 ml) reported by Boyd et al. [9], an 93.5–111.6% recovery of CPT and

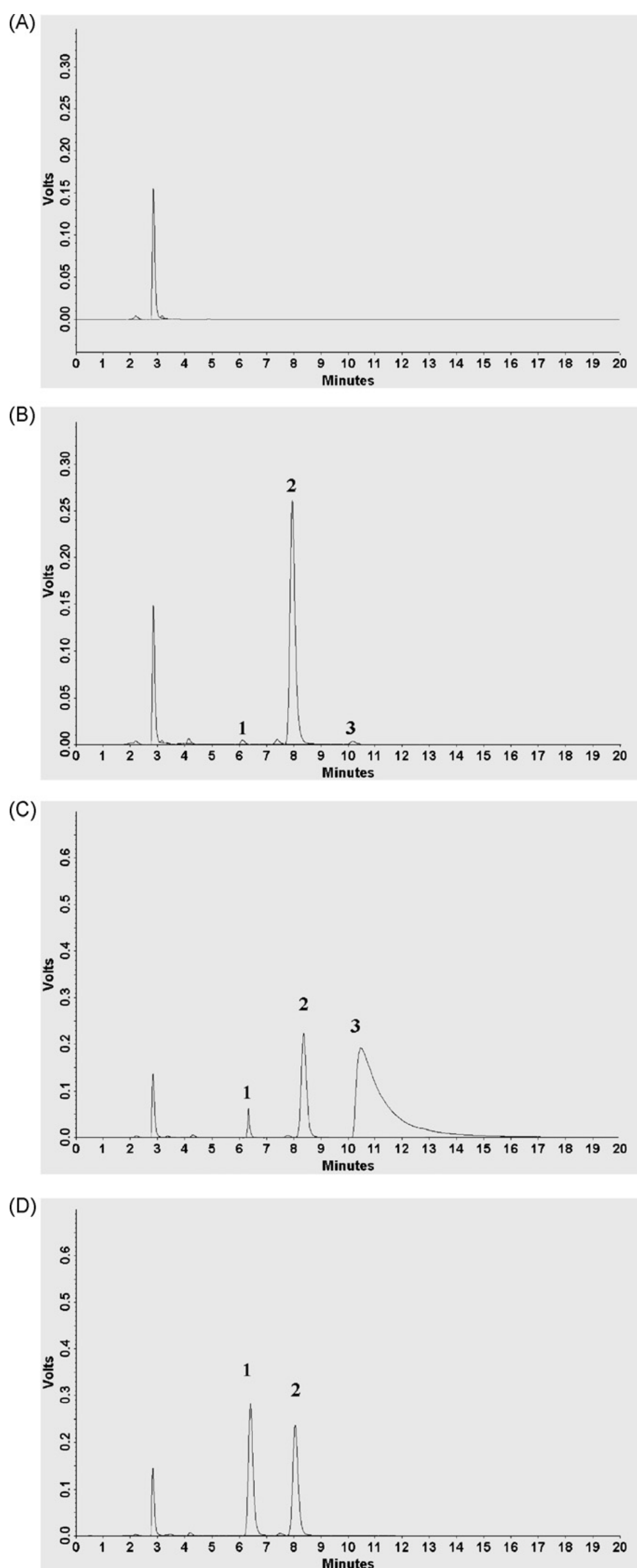

Fig. 2. Respective chromatograms of (A) blank DMEM medium sample; (B) a spiked DMEM medium sample (CPT-11 at 5 ng/ml; SN-38 at 1 ng/ml; and CPT at 1 μg/ml); (C) culture media collected 5 min after incubation of H4-II-E cells with CPT-11 (20 μM, i.e. 6968 ng/ml); (D) culture media collected 5 min after incubation of H4-II-E cells with SN-38 (1 μM, i.e. 392.4 ng/ml). Peak 1, SN-38; Peak 2, CPT; and Peak 3, CPT-11.

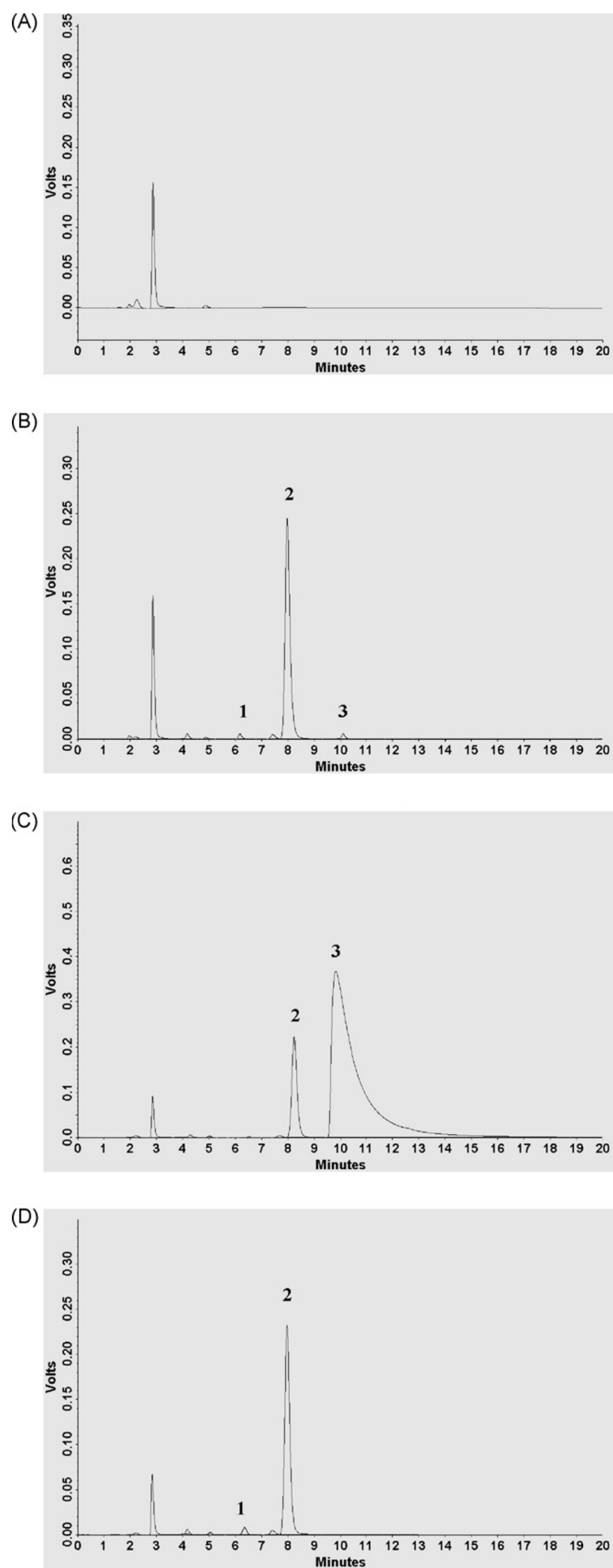

Fig. 3. Respective chromatograms of (A) blank cell lysates sample; (B) a spiked cell lysates sample (CPT-11 at 2 ng/ml; SN-38 at 0.5 ng/ml; and CPT at 1 μg/ml); (C) cell lysates collected 30 min after incubation of H4-II-E cells with CPT-11 (20 μM, i.e. 6968.0 ng/ml); and (D) cell lysates collected 5 min after incubation of H4-II-E cells with SN-38 (1.0 μM, i.e. 392.4 ng/ml). Peak 1, SN-38; Peak 2, CPT; and Peak 3, CPT-11.

Table 1
Recovery (%) of analytes from culture media and cell lysates (expressed as mean ± S.D.)

Concentration (ng/ml)	No. of sample (n)	Recovery (%)	
		Culture media	Cell lysates
CPT-11			
2 or 5	5	100.6 ± 2.1	107.2 ± 4.1
10	5	102.8 ± 7.3	102.0 ± 3.7
100	5	96.9 ± 1.8	94.3 ± 2.5
1000	5	103.2 ± 1.2	97.6 ± 1.8
10,000	5	108.3 ± 1.3	103.0 ± 3.9
42,000	5	108.3 ± 0.9	103.4 ± 0.1
SN-38			
0.5 or 1	5	87.7 ± 10.4	90.1 ± 4.7
5	5	98.3 ± 9.4	93.8 ± 3.5
20	5	101.9 ± 8.0	94.4 ± 8.7
100	5	102.1 ± 6.7	96.0 ± 3.4
500	5	106.8 ± 7.9	96.3 ± 6.9
1500	5	104.8 ± 2.5	105.6 ± 2.9

SN-38 lactones over 1–400 ng/ml was achieved from cell culture medium; and a greater variability (59.6–110.3%) was observed with the CPT and SN-38 carboxylates over 1–400 ng/ml. The simple one-step extraction method used in our study achieves quantitative recovery of both CPT-11 and SN-38 from culture media and cell homogenates. The recovery of the IS, CPT, determined at the concentration used was 90.4–103.2 and 93.2–98.7% ($n = 5$) from culture media and cell lysates/sonicates, respectively.

Assay specificity for CPT-11 and SN-38 determination was indicated by the absence of interfering chromatographic peaks in cancer cell lysates/sonicates and tissue culture media in the presence of potentially combined drugs such as thalidomide, cyclophosphamide, 5-flurouracil, nifedipine, probenecid, MK-571, or verapamil.

Both compounds gave linear response as a function of concentration over 5–42,000 ng/ml for CPT-11 and 1–1500 ng/ml for SN-38 in culture medium while 2–42,000 ng/ml for CPT-11 and 0.5–1500 ng/ml for SN-38 in cell lysates. The mean correlation coefficients (r) for the daily calibration curves were all >0.998 ($n = 6$) and the within- and between-run CVs of the response factors for each concentration assayed were below 10%. For each point on the calibration curves, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±20%. A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero. The distribution of the residuals showed random variation, was normally distributed and centered on zero. The bias was not statistically different from zero, and the 95% confidence intervals included zero (data not shown).

The LOQ values were 5.0 and 1.0 ng/ml for CPT-11 and SN-38 in culture media; and 2.0 and 0.5 ng/ml for CPT-11 and SN-38 in cell lysates, respectively. Such sensitivity ensured the determination of CPT-11 and SN-38 at lower concentrations that are often achieved in clinical settings. Boyd et al. [9] reported on the LOQ was 0.2 ng/ml for CPT lactone, 0.5 ng/ml for SN-38 lactone; and 2 ng/ml for CPT and SN-38 carboxylate. The LOQ values of our method were comparable to those reported by Boyd et al. [9]. The limit of detection was 1.0 and 0.25 ng/ml for CPT-11 and SN-38 in culture media; and 0.5 and 0.2 ng/ml for CPT-11 and SN-38 in cell lysates, respectively.

Table 2
Within- and between-day precision and accuracy of the HPLC methods for the determination of CPT-11 and SN-38 in culture media and cell lysates

Concentration (ng/ml)	Culture media		Cell lysates	
	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Within-day				
CPT-11				
2/5	4.3	95.0	4.8	92.4
10	9.6	103.9	6.5	102.3
100	4.2	110.3	7.9	90.8
1000	3.1	98.4	5.3	102.6
10,000	4.9	91.5	4.2	95.6
42,000	3.4	102.4	6.0	110.1
SN-38				
0.5/1	3.6	106.4	4.8	98.9
5	4.5	97.5	6.5	90.5
20	7.6	108.1	7.9	102.4
100	2.3	98.7	5.3	99.3
500	3.8	102.4	4.2	98.1
1500	1.2	92.9	6.0	103.2
2500	4.7	93.6	2.9	101.7
Between-day				
CPT-11				
2/5	6.3	98.4	4.6	102.5
10	3.8	100.3	3.7	88.9
100	2.0	98.0	2.9	100.8
1000	1.1	97.8	4.5	110.4
10,000	1.3	104.6	3.8	102.7
42,000	1.0	109.1	3.8	103.8
SN-38				
0.5/1	7.4	105.7	9.1	107.7
5	8.5	96.8	6.6	101.6
20	8.9	95.9	9.7	104.1
100	3.4	107.7	9.6	98.6
500	6.4	93.9	3.2	96.3
1500	5.6	92.7	7.8	103.2
2500	4.9	90.5	7.1	110.3

The validation data in terms of within- and between-day precision and accuracy are represented in Table 2. The differences between the theoretical and the actual concentration and the CVs varied from 0.1 to 10.3% at any quality control sample concentrations including the high concentration for SN-38 (2500 ng/ml) which needs 1:10 dilution.

The validated method has been applied to investigate cellular metabolism and accumulation of CPT-11 and SN-38 in H4-II-E rat hepatoma cell line. The metabolism of CPT-11 and SN-38 in H4-II-E cells incubated with DMEM with regard to incubation time and substrate concentration is shown in Fig. 4. Both SN-38 and SN-38G were detectable and achieved peak levels after 5 min incubation of CPT-11 with H4-II-E cells; thereafter, the formation of both metabolites rapidly declined over 120 min. The formation of both SN-38 and SN-38G in H4-

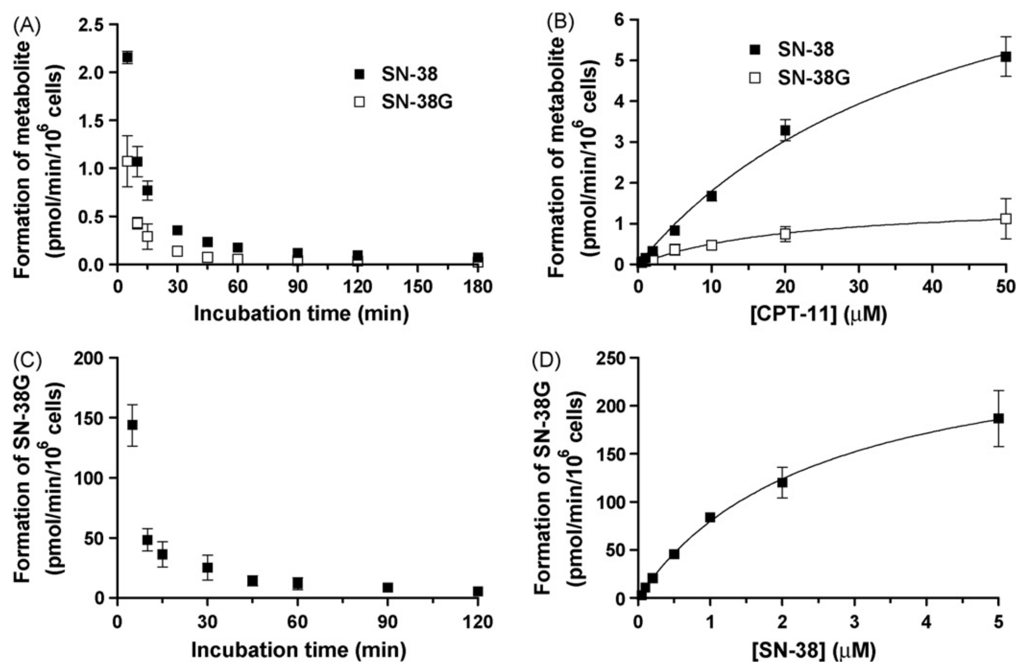

Fig. 4. Effects of incubation time and substrate concentration on the hydrolysis of CPT-11 (A and B) and SN-38 glucuronidation (C and D) in H4-II-E cells in culture medium. The curves in plots B and D represent the best fit of one-binding site model.

II-E cells increased with increasing CPT-11 concentration and followed Michaelis–Menten kinetics with the one-binding site model being the best fit. The estimated K_m and V_{max} for SN-38 formation in DMEM were 43.80 μM and 9.68 pmol/min/10^6 cells, respectively. For SN-38G formation, the K_m and V_{max} were 21.22 μM and 1.59 pmol/min/10^6 cells, respectively. In addition, when SN-38 was incubated with H4-II-E cells, SN-38G appeared rapidly in the medium and then declined over 120 min. The formation of SN-38G increased in a substrate concentration-dependent manner and followed Michaelis–Menten kinetics. One-binding site model was the best fit for the formation of SN-38G with K_m 2.50 μM and V_{max} of 278.80 pmol/min/10^6 cells.

In conclusion, we reported herein on a simple and sensitive HPLC method for simultaneous determination of CPT-11 and its major metabolite SN-38 in culture media and cell lysates. By the means of incorporation of CPT (I.S.) to samples in acidified methanol solution, we condensed the three phases in just one step: addition of I.S., acidification of sample and protein precipitation, so that the assay gains simplicity and efficiency. The separation was carried out on a reversed-phase C_{18} column and monitored at appropriate excitation and emission wavelengths at which both compounds have good response. This method was successfully applied to quantitate the cellular accumulation and metabolism of CPT-11 and SN-38 in H4-II-E cells.

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