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Liquid chromatographic–tandem mass spectrometric assay for the simultaneous quantification of Camptosar® and its metabolite SN-38 in mouse plasma and tissues

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Abstract

A simple, rapid and sensitive LC–MS/MS bioanalytical method has been developed to simultaneously quantify Camptosar (CPT-11) and its active metabolite, SN-38, in mouse plasma and tissues. A single step protein precipitation with acetonitrile in 96-well plates was used for sample preparation. Camptothecin (CPT) was used as the internal standard. Fast separation of SN-38, CPT-11 and CPT was carried out isocratically on a C_{18}, 2 mm × 50 mm, 5 μm HPLC column with a mobile phase containing acetonitrile and 20 mM ammonium acetate (pH 3.5) and a 2.5 min chromatographic run time. The API 4000 MS/MS system was operated in positive ionization multiple reaction monitoring mode, and the transitions for SN-38, CPT-11 and CPT were 393.4 → 349.3, 587.6 → 167.2 and 349.3 → 305.3, respectively. The SN-38 and CPT-11 concentrations in samples were calculated from a standard curve of peak area ratios of the analyte to that of the internal standard using a $1/x^2$ weighted linear regression. The quantitation limit of 0.5 ng/mL was achieved by using a low sample volume (100 μL) of plasma or tissue homogenates. The assay was linear over the concentration range of 0.5–500 ng/mL with acceptable precision and accuracy. The method was used for the quantification of CPT-11 and SN-38 in plasma and tissues to support a preclinical pharmacokinetics and tissue distribution study of CPT-11 in mice.

Keywords: Liquid chromatography; Tandem mass spectrometry; Camptothecin; CPT-11; SN-38; Pharmacokinetics; Mice

1. Introduction

Camptosar® and SN-38 are among a unique class of anticancer agents and are derivatives of camptothecin, a plant alkaloid which was first discovered in early 1960s [1]. The mechanism of action of camptothecins is based on the inhibition of topoisomerase I, which is a nuclear enzyme that plays a critical role in DNA replication and transcription [2,3]. Camptosar (irinotecan, CPT-11), {7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin} is a semi-synthetic and water-soluble derivative of camptothecin, CPT (Fig. 1), that exhibits improved antitumor activity and decreased toxicity [1,4,5]. CPT-11 has shown clinical activity against colorectal, lung, ovarian cancers and other malignances [6,7]. CPT-11 acts as a prodrug in vivo and is converted to its active metabolite, SN-38, {7-ethyl-10-hydroxycamtothecin} (Fig. 1) by various enzyme systems, primarily carboxyl esterase [8–10]. SN-38 has 100–1000 times greater in vitro cytotoxic activity than that of the parent compound [11,12]. Despite its promising anti-tumor activity, SN-38 cannot be used as an anti-cancer drug because of its poor solubility in pharmaceutically acceptable solvents. This technical barrier can be eliminated using a liposome entrapped SN-38 formulation (LE-SN38) [13].

LE-SN38 was very effective towards inhibiting tumors in various xenograft models of human cancer in SCID mice and showed a better preclinical safety profile [13].

A simple, rapid and sensitive bioanalytical method was needed to simultaneously quantify CPT-11 and SN-38 in plasma and tissues to support pharmacokinetics and tissue distribution studies in mice.

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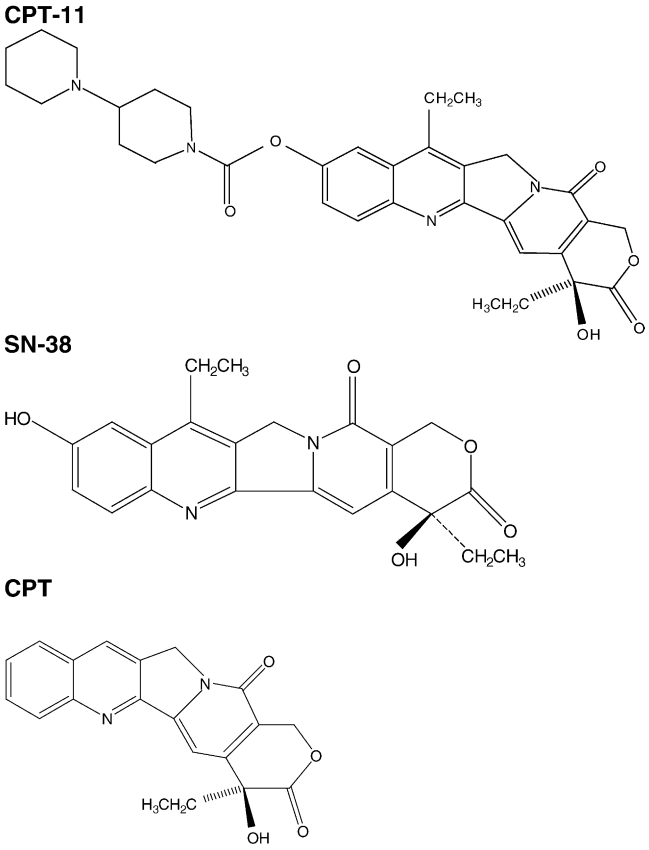

Fig. 1. Chemical structures of CPT-11, SN-38 and CPT (IS).

Previously reported assays for the determination of CPT-11 and SN-38 in biological fluids suffered from lengthy sample preparation time using off-line and on-line solid-phase extraction [14–16] or liquid–liquid extraction [14,17–19]. Several methods of physiological sample treatment, including the deproteinization step for sample clean-up, have been described in the literature [14,19,20]. These HPLC methods with fluorescence detection have chromatographic run times ranging from 8 to 20 min [15,17,21,22]. Also, relatively high sample volumes (200–1000 μL of plasma) were typically used for the reported assays to achieve better sensitivity [15,19,22,23].

Lately, LC–MS/MS technology has become the method of choice for a number of studies due to a unique combination of high specificity, sensitivity and high sample throughput possibilities. Nevertheless, the number of methods that have been developed for CPT-11 and SN-38 based on this technology are still very limited [17,24,25]. Recently, a sensitive and rapid LC–MS/MS method for the quantification of SN-38 in human plasma has been developed and implemented to support clinical studies [26].

We have developed an LC–MS/MS method for simultaneous quantification of CPT-11 and SN-38 in plasma and tissue homogenates with high selectivity and a lower limit of quantitation (LLOQ) of 0.5 ng/mL for both analytes. Sample preparation involves only a single protein precipitation step in a 96-well plate format, with automation performed using a Tomtec Quadra-96 liquid handling system for high sample throughput. Fast separation of CPT-11, SN-38 and internal standard, CPT, was achieved within 2.5 min of chromatographic run time. The method was used for CPT-11 and SN-38 quantification in plasma and tissue samples obtained from pharmacokinetic and tissue distribution studies in mice.

2. Experimental

2.1. Chemicals and reagents

Mouse plasma was obtained from Bioreclamation (Hicksville, NY, USA). Normal tissues from CD2F1 mice were collected in house. High-purity water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Acetonitrile and methanol were HPLC grade and were purchased from EM Science (Gibbstown, NJ, USA). Ammonium acetate was obtained from Fisher Scientific (Hampton, NH, USA). Camptothecin (CPT), glacial acetic acid, and dimethyl sulfoxide (DMSO) were purchased from the Sigma (St. Louis, MO, USA). Camptosar (Irinotecan hydrochloride injection, 20 mg CPT-11/mL) was purchased from Pharmacia & Upjohn (now Pfizer), (Kalamazoo, MI, USA). SN-38 was obtained from Qventas (Newark, DE, USA).

2.2. Standards and quality control samples

Standard and quality control (QC) primary stock solutions of 2 mg SN-38/mL in DMSO and 1 mg CPT-11/mL in methanol were prepared separately by dissolving the appropriate amount of each compound in known volumes of solvent.

Spiking solutions containing both CPT-11 and SN-38 at concentrations of 10, 20, 100, 200, 1000, 2000, 4000, 8000, 10 000, and 20 000 ng/mL were prepared by serial dilution of stock solutions with acetonitrile–20 mM ammonium acetate, pH 3.5 (20:80, v/v). Standards and QC samples of SN-38 and CPT-11 in plasma or tissue homogenates [5% (w/v) tissue homogenate in water] were prepared fresh for each analytical run by spiking the respective matrices with a solution containing appropriate concentrations of SN-38 and CPT-11. A stock solution of CPT, the internal standard (IS), at 0.5 mg/mL was prepared in DMSO–methanol (50:50, v/v). Standards and QCs were combined with IS and processed by protein precipitation as described in the following section.

2.3. Preparation of standards, QCs and samples (plasma and tissue homogenates) for analysis

A single step protein precipitation technique was used for sample preparation. Two hundred μL of a 10 ng/mL solution of CPT (IS) in acetonitrile were added to each well of a 96-well plate. A 100 μL volume of sample, standard or QC (plasma or tissue homogenate) was then transferred in the ap-

propriate well containing IS. The plate was covered and vortex mixed for 1–2 min, then centrifuged for 30 min at 6100 g. Using a Tomtec Quadra-96 liquid handling system [Model 320, Tomtec (Hamden, CT, USA)], the supernatants were transferred to a clean 96-well plate, and dried under nitrogen at approximately 50 °C. The samples were reconstituted with 100 μL of a mixture of acetonitrile–20 mM ammonium acetate, pH 3.5 (20:80, v/v). The plate was then covered, vortex mixed and centrifuged again for 30 min at 6100 g. The samples were kept at 4 °C before and prior to injection onto the LC–MS/MS system.

2.4. Chromatographic conditions

An Agilent 1100 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, degasser, and thermostated autosampler was used. Chromatographic separation was achieved by using either a Zorbax SB-phenyl (2.0 mm × 50 mm, 5 μm) analytical column (Agilent Technologies, Wilmington, DE, USA) with a mobile phase of 20 mM ammonium acetate, pH 3.5–acetonitrile (65:35, v/v) or an XTerra C_{18} (2.0 mm × 50 mm, 5 μm) analytical column (Waters, Milford, MA, USA) with a mobile phase 20 mM ammonium acetate, pH 3.5–acetonitrile (67:33, v/v). The analytical column was protected by a 2 μm in-line filter frit (Upchurch Scientific, Oak Harbor, WA, USA). The flow rate was 0.2 mL/min, and the run time was 2.5 min.

2.5. Mass spectrometric conditions

An Applied Biosystems-Sciex API 4000 (Applied Biosystems, Foster City, CA, USA) triple quadrupole mass spectrometer equipped with TurboIonSpray® ionization interface was used. Tuning and calibration of the mass analyzer was performed by infusing poly(propylene glycol) calibration solution (Applied Biosystems) at 10 μL/min and monitoring eight mass-to-charge ratios in the range of 55–2300 u. The peaks were tuned to 0.7 ± 0.1 u FWHM, and the calibration tolerance was ±0.1 u. Samples were analyzed in multiple reaction monitoring (MRM) positive ionization mode. Instrumental details for quantification of selected ions of CPT-11, SN-38 and IS in plasma and tissue samples are described in Table 1. The Analyst 1.2 software (Applied Biosystems) was used to control the LC–MS/MS system and to perform analyses. The peak area ratios of CPT-11 or SN-38 to those of the IS were used to construct a linear calibration curve using weighted (1/concentration^2) regression analysis. The CPT-11 and SN-38 concentrations in plasma and tissue samples were determined by interpolation from the calibration curve.

Table 1
Instrument parameters and transitions for SN-38, CPT-11 and IS (CPT)

Parameter	Value
Temperature (°C)	550
Ionization voltage (V)	5000
Ion source (GS1) setting	20
Ion source (GS2) setting	40
Curtain gas settings	10
CAD gas setting	8
Collision energy (IS, SN-38) (eV)	35
Collision energy (CPT-11) (eV)	55
SN-38 transition	393.4 → 349.3
IS transition	349.3 → 305.3
CPT-11 transition	587.6 → 167.2

2.6. Ruggedness of the method

Accuracy and precision were assessed by analyzing QC samples. Ruggedness data have been generated separately for CPT-11 and SN-38. Each ruggedness run included a calibration curve with eight non-zero standards and four concentration levels of QC samples in triplicate. Assay precision was calculated and reported as the percent relative standard deviation (R.S.D.). Accuracy was determined as an agreement between the interpolated concentration and the nominal concentration and was reported as the percent analytical recovery (AR). Acceptance criteria for precision were set at ≤15% R.S.D., while those for analytical recovery (AR) were set at 100 ± 15% [27].

3. Results and discussion

3.1. Assay development

MS and MS/MS conditions were optimized for CPT-11, SN-38, and IS through infusion to ascertain the precursor ion, and then to select the product ions in MS/MS mode.

Table 2
Precision and accuracy for CPT-11 and SN-38 quantification in mouse plasma

Nominal concentration (ng/mL)	SN-38			CPT-11		
	Mean[a] measured concentration (ng/mL)	Precision, R.S.D. (%)	Accuracy, AR (%)	Mean[a] measured concentration (ng/mL)	Precision, R.S.D. (%)	Accuracy, AR (%)
1.50	1.31	2.71	87.0	1.44	12.7	96.0
3.00	3.35	7.13	112	2.99	4.62	100
30.0	31.0	4.85	103	29.8	3.81	99.4
300	289	2.31	96.2	295	0.781	98.4

R.S.D., precision expressed as relative standard deviation (standard deviation/mean result) × 100; AR, accuracy expressed as analytical recovery (mean result/nominal concentration) × 100.

[a] Three replicates at each concentration.

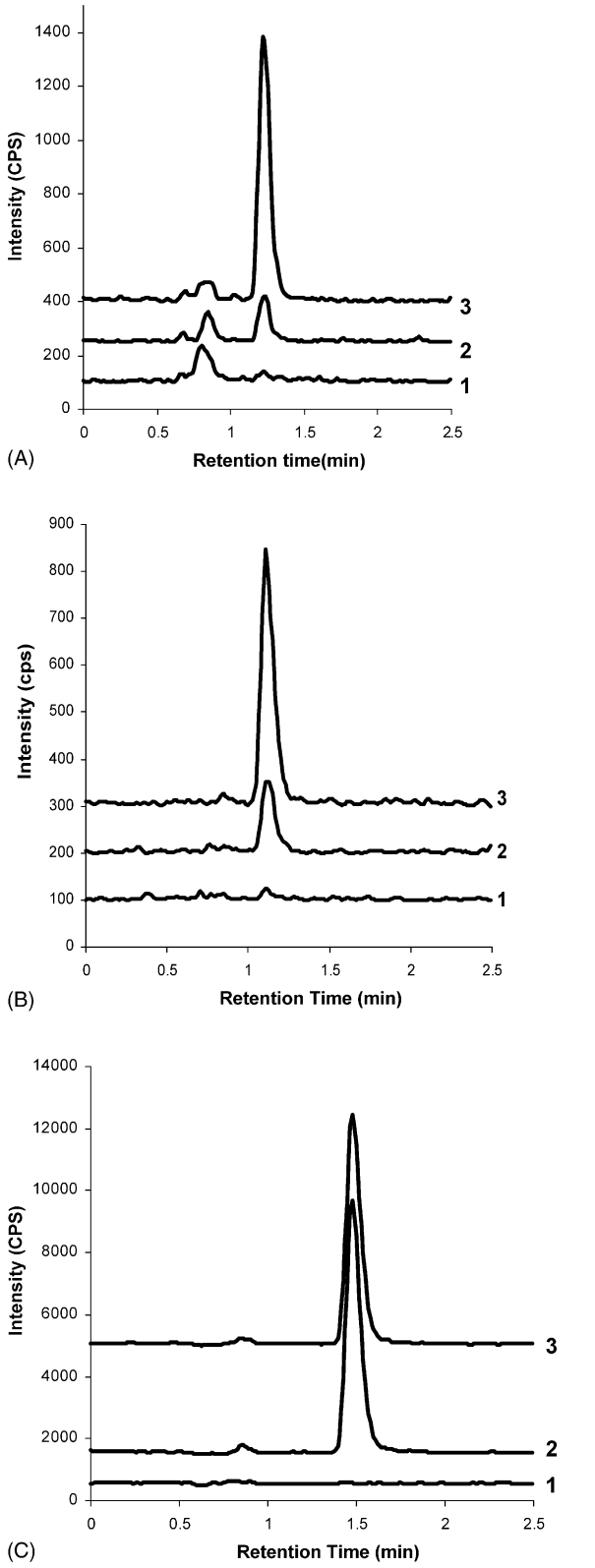

Fig. 2. Representative chromatograms of mouse plasma extracts containing CPT-11, SN-38 and CPT (IS). (A) SN-38 channel; (B) CPT-11 channel; (C) CPT channel. 1: Blank plasma sample; 2: standard, 0.5 ng/mL; 3: QC sample, 1.5 ng/mL.

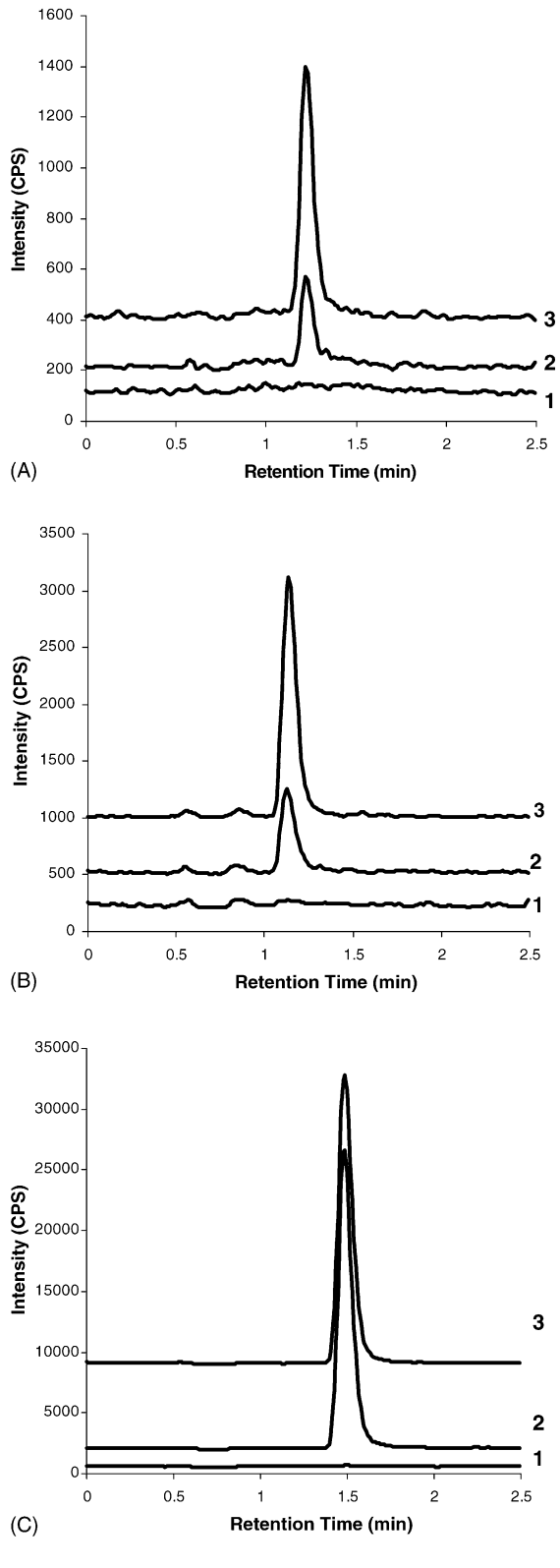

Fig. 3. Representative chromatograms of spiked mouse tissue homogenate (intestine) extracts containing CPT-11, SN-38 and CPT (IS). (A) SN-38 channel; (B) CPT-11 channel; (C) CPT channel. 1: Blank matrix sample; 2: standard, 0.5 ng/mL; 3: QC sample, 1.5 ng/mL.

Table 3
Precision and accuracy for CPT-11 and SN-38 quantification in mouse tissue homogenates

Tissue	Nominal concentration (ng/mL)	SN-38			CPT-11		
		Mean[a] measured concentration (ng/mL)	Precision, R.S.D. (%)	Accuracy, AR (%)	Mean[a] measured concentration (ng/mL)	Precision, R.S.D. (%)	Accuracy, AR (%)
Kidney	1.50	1.54	5.55	103	1.69	1.90	113
	3.00	3.16	4.11	105	3.34	2.29	111
	30.0	31.2	2.59	104	32.6	0.812	109
	300	291	1.30	97.1	283	1.54	94.3
Liver	1.50	1.31	2.46	87.1	1.48	3.04	99.0
	3.00	2.98	5.03	99.2	3.12	5.19	104
	30.0	30.2	1.56	101	30.0	2.70	99.9
	300	289	0.200	96.4	278	0.360	107
Spleen	1.50	1.54	11.0	103	1.52	10.2	101
	3.00	3.23	3.14	108	2.78	0.623	92.7
	30.0	31.3	1.76	104	28.3	1.09	94.2
	300	307	1.05	102	307	3.01	102
Lung	1.50	1.47	5.77	98.0	1.59	2.67	106
	3.00	3.06	6.69	102	3.17	2.55	106
	30.0	29.7	2.43	99.0	31.8	0.831	106
	300	308	1.50	103	301	0.879	100
Heart	1.50	1.53	3.10	102	1.49	1.94	99.1
	3.00	3.14	5.42	105	3.07	5.43	102
	30.0	31.8	3.50	106	32.5	1.86	108
	300	318	1.10	106	319	0.830	106
Intestine	1.50	1.68	2.82	112	1.69	3.31	107
	3.00	3.18	3.63	106	3.26	2.45	109
	30.0	30.6	1.32	102	33.2	4.44	111
	300	294	0.392	98.1	286	0.700	95.3
Tumor	1.50	1.66	5.25	111	1.56	4.18	104
	3.00	3.12	3.89	104	3.09	2.79	103
	30.0	31.4	4.31	105	32.3	1.12	108
	300	309	1.41	103	308	1.46	103

[a] Three replicates at each concentration.

The following transitions were identified to be optimal for analysis of the analytes and IS: SN-38: 393.4 → 349.3; CPT-11: 587.6 → 167.2; CPT: 349.3 → 305.3 (Table 1). HPLC conditions were developed to optimize the separation for CPT-11 and SN-38 peak shape, resolution, selectivity and sensitivity.

Two HPLC columns were evaluated for the analysis of CPT-11 and SN-38 in both plasma and tissue homogenates: a Zorbax SB-phenyl 2 mm × 50 mm, 5 μm column with a mobile phase composition of 20 mM ammonium acetate, pH 3.5–acetonitrile ratio of 65:35 (v/v), and an XTerra C_{18} 2 mm × 50 mm, 5 μm column using the same mobile phase with buffer–acetonitrile ratio of 67:33 (v/v). Both columns were found to be suitable for the application, producing good peak shapes and sufficient retention of the analytes and IS. However, the XTerra C_{18} column provided better peak shape for SN-38 in samples with CPT-11 concentration 100 times or more higher than that of SN-38. The method sensitivity limit was 0.5 ng/mL for both analytes. The LC–MS quantification method described in [17] had a sensitivity of 10 ng/mL and 0.5 ng/ml in human serum for CPT-11 and SN-38, respectively. In this case, the two analytes had to be assayed separately when the level of CPT-11 was 100 times higher than that of SN-38.

3.2. Assay selectivity, linearity, accuracy and precision

Representative chromatograms for plasma and tissue homogenates without analytes (blanks), LLOQ standards and low level QC samples are shown in Figs. 2 and 3, respectively, for SN-38, CPT-11 and CPT (IS) channels. As can be seen from these figures, no significant interfering peaks were observed at the retention times of the analytes and/or internal standard.

Standard curves were established by plotting the peak area ratio of SN-38 or CPT-11 to that of the IS versus nominal concentration of the analyte. The resulting linear-regressions yielded correlation coefficients ≥0.995 for all runs, and the range of linearity was 0.5–500 ng/mL for both CPT-11 and SN-38 in plasma and tissue homogenates. Assay results from QC samples showed acceptable precision and accuracy. In plasma, the R.S.D. ranges for CPT-11 and SN-38 were

from 0.781 to 12.7% and from 2.31 to 7.13%, respectively (Table 2). The AR ranged from 96.0 to 100% and from 87.0 to 112% for CPT-11 and SN-38, respectively (Table 2). In tissue homogenates, the R.S.D. and the AR met acceptance criteria for both CPT-11 and SN-38 at all tested concentrations (Table 3).

3.3. Assay application

Recently, the number of studies using LC–MS technology in the field of pharmacokinetics and clinical toxicology has significantly increased [14,28]. At the same time only few LC–MS/MS methods had been published for the simultaneous quantification of both CPT-11 and SN-38 [17,24,25]. An HPLC–atmospheric pressure chemical ionisation (APCI) MS method was used in clinical studies primarily for identification of photodegradation products of CPT-11 [24]. An HPLC method with parallel fluorescence and mass spectrometric detection of CPT-11, SN-38 and other metabolites of CPT-11 in human plasma has been also described [25]. This method has achieved the quantification of CPT-11 and SN-38 with sensitivities of 5 and 1 ng/mL, respectively, using fluorescence detection, while mass-spectrometric detection was used for identification and monitoring of CPT-11 metabolites. An LC–MS/MS method with an improved sensitivity of 0.05 ng/mL for SN-38 was used to support clinical studies for LE-SN38 [26]. The method presented here was successfully used to quantify CPT-11 and SN-38 in plasma and tissue samples from a pharmacokinetics and tissue distribution study of CPT-11 in mice. The representative concentration–time profiles for CPT-11 and SN-38 in plasma and tissues following intravenous administration of 35 mg/kg of CPT-11 are presented in Figs. 4 and 5, respectively.

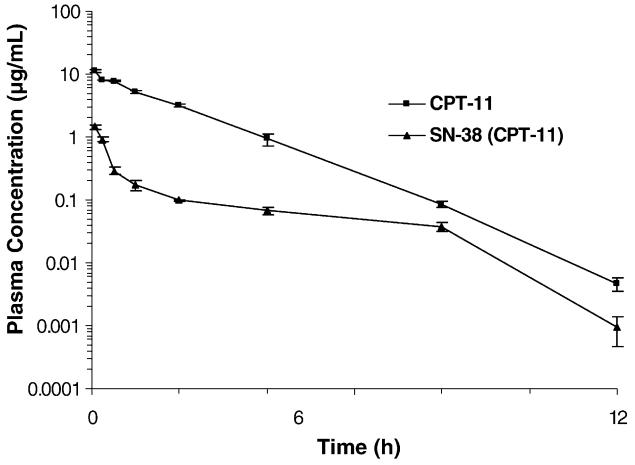

Fig. 4. Representative plasma concentration–time profiles of SN-38 and CPT-11 following i.v. administration of CPT-11 in mice at a 35 mg/kg dose level. Data points represent mean ± standard error of the mean, $n = 3$ animals per time point.

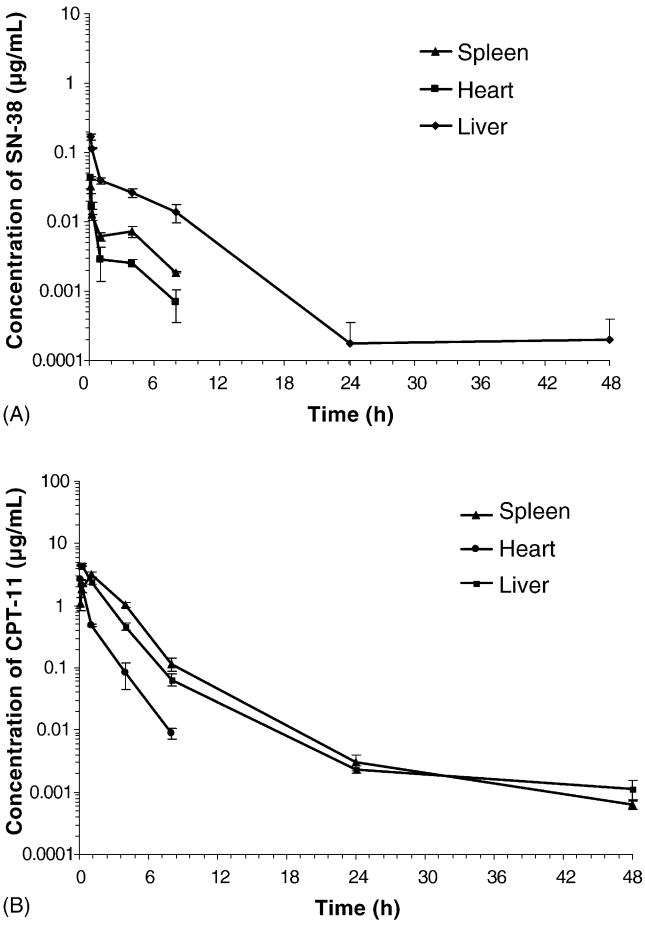

Fig. 5. Representative tissue homogenate concentration–time profiles of (A) SN-38 and (B) CPT-11 following intravenous administration of CPT-11 in mice at a 35 mg/kg dose level. Data points represent mean ± standard error of the mean, $n = 3$ animals per time point.

4. Conclusion

A simple, sensitive and rapid LC–MS/MS assay was successfully developed for the simultaneous determination of total CPT-11 and SN-38 levels in mouse plasma and tissues. The selectivity and sensitivity of tandem mass spectrometric detection enabled a short chromatographic run time of 2.5 min and a lower limit of quantitation of 0.5 ng/mL for both analytes in plasma and tissues homogenates. Sample preparation involved only a one step protein precipitation procedure which allowed high sample throughput yet gave high sensitivity using a low sample volume of 100 μL. The assay was successfully implemented for a pharmacokinetics and tissue distribution study of CPT-11 in mice. This method will be used to support pharmacokinetic and tissue distribution studies of LE-SN38 in mice.

References

[1] M.F. Wall, M.C. Wani, C.F. Cook, K.H. Palmer, A.T. McPhail, G.A. Sim, J. Am. Chem. Soc. 88 (1966) 3888.

[2] R. Garcia-Carbonero, J.G. Supko, Clin. Cancer Res. 8 (2002) 641.
[3] R.H.J. Mathijessen, R.J. Van Alphen, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, Clin. Cancer Res. 7 (2001) 2182.
[4] R.C. Gallo, J. Whang-Peng, R.H.J. Adamson, Natl. Cancer Inst. 46 (1971) 789.
[5] T. Kunimoto, K. Nitta, T. Tanaka, N. Uehara, H. Baba, M. Takeuchi, T. Yokokura, S. Sawada, T. Miyasaka, M. Mutai, Cancer Res. 47 (1987) 5944.
[6] G.J. Creemers, B. Lung, J. Verweij, Cancer Treat. Rev. 20 (1994) 73.
[7] Y.-H. Hsiang, R. Hertzberg, S. Hecht, L.F. Kiu, J. Biol. Chem. 260 (1985) 14873.
[8] T. Tsui, N. Kaneda, K. Kado, T. Yokokura, T.J. Yoshomoto, J. Pharmacobiodyn. 14 (1991) 341.
[9] T. Satoh, T. Hosokawa, R. Atsumi, W. Suzuki, H. Hakisui, E. Nagai, Biol. Pharm. Bull. 17 (1994) 662.
[10] L. Rivory, M. Bowles, J. Robert, S. Pond, Biochem. Pharmacol. 52 (1996) 1103.
[11] C.H. Takimoto, S.G. Arbuck, in: B.A. Chabner, D.L. Longo (Eds.), Cancer Chemotherapy and Biotherapy: Principles and Practice, Lippincott Williams & Wilkins, Philadelphia, 2001, p. 579.
[12] M.L. Rothenberg, J.R. Eckardt, J.G. Kuhn, H.A. Burris 3rd, J. Nelson, J.R. Eckatdt, M. Tristan-Morales, S.G. Hilsenbeck, G.R. Weiss, L.S. Smith, G.I. Rodrigez, M.K. Rock, D.D. Von Hoff, J. Clin. Oncol. 11 (1993) 2194.
[13] J.A. Zhang, T. Xuan, M. Parmar, L. Ma, S. Ugwu, S. Ali, I. Ahmad, Int. J. Pharm. 270 (2004) 93.
[14] T.-H. Tsai, J. Chromatogr. B 764 (2001) 27.
[15] L.P. Rivory, M. Findlay, S. Clarke, J. Bishop, J. Chromatogr. B 714 (1998) 355.
[16] A. Kurita, N. Kaneda, J. Chromatogr. B 724 (1999) 335.
[17] S. Ragot, P. Marquet, F. Lachatre, A. Rousseau, E. Lacassie, J.M. Gaulier, G. Dupuiy, G. Lachatre, J. Chromatogr. B 736 (1999) 175.
[18] H. Sumiyoshi, Y. Fujiwara, T. Ohune, N. Yamaoka, K. Tamura, M. Yamakido, J. Chromatogr. B 670 (1995) 309.
[19] A. Sparreboom, P. de Bruijn, M.J.A. de Jonge, W.J. Loos, G. Stotter, J. Verweij, K. Nooter, J. Chromatogr. B 712 (1998) 225.
[20] J. Escoriaza, A. Aldaz, C. Castellanos, E. Calvo, J. Giraldes, J. Chromatogr. B 740 (2000) 159.
[21] D.L. Warner, T.G. Birke, J. Chromatogr. B 691 (1997) 161.
[22] D.F. Chollet, L. Goumas, A. Renard, G. Montay, L. Vernillet, V. Arnera, D.J.J. Mazzo, J. Chromatogr. B 718 (1998) 163.
[23] P. de Bruijn, J. Verweij, W.J. Loos, K. Nooter, G. Stoter, A. Sparreboom, J. Chromtogr. B 698 (1997) 277.
[24] H.M. Dodds, J. Robert, L.P. Rivory, J. Pharm. Biomed. Anal. 17 (1998) 785.
[25] K. Sai, N. Kaniwa, S. Osawa, J.-I. Sawada, Biomed. Chromatogr. 16 (2002) 209.
[26] S. Khan, A. Ahmad, I. Ahmad, Biomed. Chromatogr. 17 (2003) 493.
[27] Food and drug administration, Center for Drug Evaluation and Research, Guidance for Industry, “Bioanalytical Method Validation”, May 2001. http://www.fda.gov/cder/guidance.
[28] P. Marquet, G. Lachatre, J. Chromatogr. B 733 (1–2) (1999) 93.