Original Research

HPLC analysis and extraction method of SN-38 in brain tumor model after injected by polymeric drug delivery system

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Abstract

SN-38 is a highly potent anticancer drug but its poor solubility in aqueous solvent and adverse side effects limit clinical applications. To overcome these limitations, SN-38-loaded-injectable drug delivery depots have been intratumorally administered in xenograft tumor model in nude mice. The extraction and high performance liquid chromatography (HPLC) were performed in order to determine the amount of SN-38 inside tumors. SN-38 was extracted from tumors using DMSO. HPLC analysis was validated and resulted in linearity over the concentration range from 0.03 to 150 μg/mL ($r^2 \geq 0.998$). Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were 0.308 μg/mL and 1.02 μg/mL, respectively. The extraction efficiency (% recovery) of SN-38 in porcine tissues was similar to that of tumors which provided more than 90% recovery in all concentrations. Moreover, the variability of precision and accuracy within and between-day were less than 15%. Therefore, this extraction and HPLC protocol was applied to determine the amount of SN-38 in tumors. Results show higher remaining amount of SN-38 in tumor from SN-38-loaded polymeric depots than that of SN-38 solution. These results reveal that SN-38-loaded polymeric depots can prevent the leakage of free-drug out of tumors and can sustain higher level of SN-38 inside tumor. Thus, the therapeutic efficacy can be elevated by SN-38-loaded polymeric depots.

Keywords: SN-38, polymeric depots, extraction, HPLC, tumor

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Introduction

7-Ethyl-10-hydroxycamptothecin, SN-38, is an extremely potent anticancer agent that used especially for brain cancer treatment.[1,2] SN-38 acts as topoisomerase enzyme inhibitor resulting in the blockage of DNA synthesis.[3] Moreover, it has more potent therapeutic efficacy and its half-life is much longer than irinotecan (CPT-11) which is its prodrug.[4,5] However, the use of SN-38 in clinical applications is limited due to its poor solubility property and many severe side effects such as diarrhea and myelo-suppression.[3] Direct injection of free drugs into tumor exhibits inherent drawbacks such as uncontrollable dose[6] and systemic toxicity[7] which may significantly limit the clinical applications. From these reports, neither sustaining release of drugs nor maintaining the therapeutic concentration for a prolonged period of time can be achieved by direct injection of free drugs. To overcome these limitations, several drug delivery systems have been developed as alternative approach for cancer chemotherapy. One strategy is to use biodegradable polymers for SN-38 delivery such as nanoparticles,[8] micelles,[9–11] liposomes,[12] or dendrimer.[4] Not only systemic drug delivery systems, but many local drug delivery systems also have been developed such as polymer millirods,[13] wafers,[14] or self-forming polymeric depots[15] for continuously controlled release of SN-38. Tremendous advantages of local implant drug delivery systems have been reported such as improvement of therapeutic efficiency, reduction of anticancer drug toxicity, and minimally invasive methods.[16,17] Recently, we reported the development of SN-38-loaded injectable drug delivery systems for gliomas using biodegradable polymers.[18] This system comprises SN-38, glycofurol (GF), and PLEC copolymers (consisted of poly(ethylene glycol) (PEG), ε-caprolactone, and D,L-lactide). The preparation and characterization of these systems *in vitro* including encapsulation efficiency, drug release, cytotoxicity, and degradation were successfully reported.[18] Moreover, Nasongkla et al.[19] and Boongird et al.[20] also reported biocompatibility of these polymeric depots in rat brain.

In this study, SN-38 containing PLEC polymeric depots were injected intratumorally to evaluate the antitumor

efficacy. The amount of SN-38 inside tumor tissues was determined by efficient extraction which is crucial for accuracy, precision of biological samples and also impacts the quantitative and qualitative of analysis. Many researchers have reported the extraction of SN-38 in biological fluids and tissues such as heart, liver, spleen, kidney, and tumor, etc. that are associated with systemic drug delivery.[21,22] Table 1 summarizes extraction techniques for the analysis of CPT-11 and metabolites in the literature. In some cases, the extraction of drug from biological matrix required several steps and is time consuming.[23] Moreover, most of tissue extractions were similar to sample preparation method for plasma such as using organic solvent,[24] protein precipitation,[25] or solid phase extraction,[24] and so on. With different physiological composition from plasma, tissues and tumors contain higher hydrophobic composition than plasma. Thus, large interferences could be co-extracted.[26] Accordingly, the suitable extraction method for analysis of SN-38 in tissues should be modified and established to determine the amount of SN-38 in tumors. In this study, SN-38 solution and SN-38-loaded polymeric depots were injected into tumors and then extraction of SN-38 from tissues and high performance liquid chromatography (HPLC) analysis was investigated to determine the amount of SN-38 in tumors. Moreover, the intratumoral dose of SN-38 from solution and polymeric depot administration was compared. Results from this study provide valuable information on the determination of residual SN-38 inside tumors which will be very useful for the design of intratumoral drug delivery systems.

Materials and methods

Materials

SN-38 was obtained from Abatra Technology Company Ltd. (Xi'an, Shaanxi, China). Glycofurol was purchased from Sigma-Aldrich (St. Louis, MO, USA). PLECs were polymerized and sterilized as described in the previous reports.[15,18,19] Dimethyl sulphoxide (DMSO), phosphoric acid, chloroform, hexane, tetrahydrofuran (THF), acetonitrile, and methanol (HPLC grades) were purchased from RCI Labscan Ltd. (Milwaukee, WI, USA). Sodium phosphate monobasic ($NaH_2PO_4\ H_2O$) was obtained from Mallinckrodt Baker, Inc. (St. Louis, MO, USA). Water used in this study was deionized in TKA water purification system (Niederelbert, Germany).

Methods

HPLC conditions. HPLC system consisted of 2695 separations module, 2489 UV-visible detector and Empower software all from Waters Ltd. (Milford, MA, USA). Chromatographic separation of SN-38 was carried out using C_{18}, SunFire column (4.6 × 100 mm i.d., 3.5 μm particle size), preceded by guard column Waters Ltd. The mobile phase comprised of 25 mM $NaH_2PO_4\ H_2O$ which was adjusted to pH 3.1 using phosphoric acid and acetonitrile solution (70:30, v/v %). In all instances, an injection volume was 10 μL and a flow-rate of 1.0 mL/min was employed. The eluted SN-38 peak was monitored at 380 nm.

Preparation of polymeric depots. The mixture of protonated SN-38 and PLEC was prepared by dissolving 15 mg of protonated SN-38 and 35 mg of PLEC in 100 μL of GF.[18] For solution, SN-38 was dissolved by GF and drawn into the syringe (26 G).

Preparation of stock and standard solution. Stock solution containing 1, 10, and 60 mg/mL of SN-38 in DMSO was prepared as low, medium, and high concentration for quality control (QC), respectively. The stock solution was diluted into sequentially working solution by DMSO to prepare the standard calibration curve.[27] Both stock and working solutions were freshly prepared and immediately injected into HPLC to confirm the quality of SN-38 before use.

Preparation of QC samples

QC samples were prepared in order to study the extraction and HPLC protocols of SN-38 in tumors. Samples were sliced to 12.5 × 12.5 × 12.5 mm^3 (1953.125 mm^3) thickness, placed on a Petri dish and weight of each slice was controlled in the range from 1.8 to 2.0 g. Then, the stock solution was gently injected into samples through a 1 mL syringe with 26 G needle and placed at room temperature until drug diffused over the samples. Stock solutions contained low, medium, and high amount of SN-38 in DMSO were prepared as described above. Low and medium amount of SN-38 (0.1 and 1 mg) in samples were prepared by injecting 100 μL of SN-38 stock solution into samples while the injection volume of 250 μL was for high amount of SN-38 (15 mg). After that, samples were kept at −80°C and the leakage of SN-38 outside the samples was measured. The leaked SN-38 on Petri dish was dissolved by 2 mL of DMSO. The solution was then centrifuged for 5 min at 10°C, 4000 rpm. The supernatant was used to determine the amount of SN-38 at its maximum absorption wavelength (λ_{max}) at 384 nm by UV-visible spectroscopy. Finally, the total amount of SN-38 in samples was calculated by subtracting the amount of leaked SN-38 outside samples from the amount of SN-38 from the stock solution that was injected into each sample. Due to the high number of samples required, porcine tissues were used as QC samples.

Extraction conditions of SN-38 from QC samples

Protocol A-D. QC samples ($n = 3$) with low amount of SN-38 were prepared as previously described. They were rinsed three times with distilled water and carefully cut into small pieces in order to increase the surface area. After that, 10 mL of different solvents (Table 2) was added and samples were homogenized for 5 min. The extracted solution was placed on the orbital shaker to allow solvent evaporation. Then, 10 mL of DMSO was added to dissolve SN-38. The solution was centrifuged at 10°C and 4000 rpm for 5 min. The supernatant was filtered by 0.2 μm nylon filter then the absorbance was measured at 384 nm using UV-visible spectroscopy. The standard calibration curve was prepared by dissolving SN-38 in DMSO.[27]

Table 1 Extraction techniques for the analysis of CPT-11 and metabolites in the literature

Drugs	Matrix	Sample preparation	Recovery (%)	Ref
SN-38	Mice tumor (HT-29 human colon cancer), heart, kidney, liver, lung and spleen	– Frozen in liquid nitrogen – Homogenized with PBS – Extracted using dichloromethane: methanol (4:1, v/v) – Evaporated and then reconstructed by methanol	N/A	(33)
SN-38	Rats liver, spleen, kidney and lung	– Homogenized with saline – Addition of methanol:acetonitrile (1:1, v/v)	N/A	(34)
SN-38	Mice heart, kidney, liver, lung, spleen and beagle dog plasma	– Homogenized with acetonitrile and 0.5% acetic acid (5%, w/v) – Evaporated and then reconstructed by acetonitrile:ammonium acetate buffer pH 3.5 (80:20, v/v)	N/A	(30)
SN-38	Human serum	– Extracted using the mixture of 2.5 M sodium citrate (pH = 2) and diethyl ether: dichloromethane (4:1, v/v) – Evaporated and then reconstructed with acetonitrile: 5 mM ammonium formate (pH = 3) (70:30, v/v)	N/A	(30)
CPT-11 and SN-38	Mice heart, kidney, liver, lung and tumor	– Homogenized with water (5%, w/v) – Addition of acetonitrile – Evaporated and then reconstructed by acetonitrile: 20 mM ammonium acetate buffer pH 3.5 (80:20, v/v)	CPT-11: 103–108% SN-38: 103–111% (spiked)	(38)
CPT-11 and SN-38	Mice brain	– Homogenized with ammonium acetate buffer (10 mM, pH = 3.5) and zinc sulfate – Addition of acetonitrile – Evaporated and then reconstructed by ammonium acetate: acetonitrile (80:20, v/v)	CPT-11: 4.2–6.1% SN-38: 19.8–38.5% (spiked)	(39)
CPT-11	Human tumor (Peritoneal cancer), peritoneal tissue and parietal muscle from patient with peritoneal carcinoma	– Homogenized with Tris-0.1 M HCl (pH = 8.1) – Addition of acidic methanol – Evaporated and reconstructed by acetonitrile:water (36:64, v/v)	N/A	(32)
CPT-11	Rats brain and U87 tumor	– Grounded under liquid nitrogen and homogenized with water (50%, w/v) – Addition of acidic methanol (20% 0.1 M phosphoric acid and 80% methanol)	>95% (spiked)	(40)
CPT	Mice heart, liver, lung, spleen and tumor (LS174T colon carcinoma)	– Homogenized with 0.1 N HCl and lysis reagent (cellytic-MT mammalian tissue lysis reagent) (1:4, v/v) – Addition of cold methanol	N/A	(23)
CPT	Rats brain, heart, kidney, liver, lung and spleen	– Homogenized with PBS (pH = 3.0):0.15 M phosphoric acid (1:1, v/v) – Addition of acetonitrile and 0.15 M phosphoric acid (1:4, v/v)	86.4–99.9% (spiked)	(36)
HCPT	Mouse liver	– Homogenized with 1% sodium phosphate solution (1:2, w/v) – Incubated with 1% tri-sodium phosphate – Extracted using dichloromethane – Extracted again using diethyl ether – Evaporated and then reconstructed with acetonitrile	N/A	(37)
HCPT	Rats liver, kidney and feces	– Homogenized with 0.9% NaCl pH7.4 (1:5, w/v) – Incubated with extraction buffer (10% SDS, 5 M NaCl, 1 M Tris (pH 7.6), 0.5 M EDTA and proteinase K (2 mg/mL) for 2 h. – Addition of methanol acetonitrile (1:1, v/v)	75–91% (spiked)	(35)

Note: (spiked) = recovery (%) was obtained from tissue homogenate that was spiked with known concentration of analyte.
CPT: Camptothecin, CPT-11: Irrinotecan, HPCT = 10-Hydroxycamptothecin.

Table 2 Summary of solvents used in extraction protocols

Protocol	Solvent	Method	
		Solvent evaporation	Additional DMSO (mL)
A	Hexane	Yes	10
B	Acetone		
C	Chloroform–methanol (4:1, v/v)		
D	DMSO–methanol–THF (5:50:45, v/v)		
E	DMSO–PBS (pH 3.1) (3:2, v/v)	No	
F	Acetonitrile–methanol (1:1, v/v)		
G	DMSO		

Protocol E-G. QC samples ($n = 3$) were prepared as described above then they were rinsed three times with distilled water and cut into small pieces in order to increase the surface area. After that, 10 mL of different solvents (Table 2) was added and samples were homogenized for 5 min. The solution was centrifuged at 10°C and 4000 rpm for 5 min. The supernatant was filtered by 0.2 μm nylon filter then the absorbance was measured at 384 nm using UV-visible spectroscopy. The standard calibration curve was prepared by dissolving SN-38 in acetonitrile-methanol (1:1, v/v), DMSO-PBS (pH 3.1) (3:2, v/v), and DMSO.

Validation of HPLC procedures

Validation of HPLC procedures was carried out using QC samples which were prepared as described earlier. First, QC samples were rinsed three times with distilled water and carefully cut into small pieces in order to increase the surface area. After 10 mL of DMSO was added, samples were homogenized for 5 min. The solution was centrifuged at 10°C and 4000 rpm for 5 min and the supernatant was centrifuged again at 10°C and 14,000 rpm for 10 min. After that, the supernatant was filtered by 0.45 and 0.2 μm nylon filter, respectively prior to the HPLC analysis. HPLC method including selectivity, linearity, precision and accuracy were validated. These parameters were proposed by Owens et al.[28] and details of each parameter such as concentration range of QC samples or sample replication were adapted and optimized for this study as described in the following section.

Selectivity

Selectivity of this method was defined by comparing triplicate extracted samples of blank tumor tissues and tumor with spiked SN-38 stock solution.

Linearity and sensitivity (Calibration curve, LLOD, and LLOQ)

Calibration curves were constructed in triplicate from SN-38 standard which covered the concentration range from 0.03 to 150 μg/mL. The linearity of this method was determined by the relationship between concentrations of standard SN-38 and its resulting area under curve (AUC) in terms of linear regression correlation coefficient (r^2), y-intercept, extinction coefficient (slope), and relative standard deviation (RSD). Lower limit of detection (LLOD) and lower limit quantitation (LLOQ) were defined according to equations (1) and (2), respectively.

$$\mathrm{LLOD} = \frac{3\sigma}{\mathrm{S}} \tag{1}$$

$$\mathrm{LLOQ} = \frac{10\sigma}{\mathrm{S}} \tag{2}$$

$$\sigma = \sqrt{\frac{\sum (y - yi)^2}{n - 2}} \tag{3}$$

where σ is the standard deviation and s is the slope of calibration curve.

Precision and accuracy

The precision of HPLC system and extraction procedure were performed for four consecutive days by analyzing replicate QC samples at low, medium, and high amount of SN-38. QC samples for low, medium, and high amount of SN-38 were extracted and injected into the column (five injections/replicate) in one day for the evaluation of within-day precision. For between-day precision, this assay was done on three separate days. For the accuracy, OQ samples were compared with the actual amount of SN-38 as shown in equation (5). The precision was evaluated by %RSD as shown in equation (6). Moreover, both QC samples and the calibration were included during each analytical run.

$$\%\ \mathrm{Accuracy} = \frac{\text{Average amount of SN} - 38 \text{ in QC samples}}{\text{Actual amount of SN} - 38} \times 100 \tag{5}$$

$$\%\ \mathrm{RSD} = \frac{SD}{\overline{X}} \tag{6}$$

where $\overline{X}$ is the average amount of SN-38 from QC samples.

Extraction efficiency (% recovery)

The extraction efficiency of SN-38 from porcine tissues was evaluated by three concentrations of QC samples. It should be noted that the extraction efficiency of SN-38 from tumors was also carried out by injecting three concentrations of SN-38 into blank tumors. Recovery at low, medium, and high amount of SN-38 (0.1, 1, and 15 mg) in porcine and tumor samples were prepared and extracted using DMSO as described earlier. Recovery of these samples was calculated by comparing the AUC of active SN-38 which was obtained from spiking the same amount of SN-38 solution into the

extracted solution of blank porcine and tumor samples as shown in equation (4).

$$\%\ \text{Recovery} = \frac{\text{Amount of SN}-38\ \text{from extraction (mg)}}{\text{Amount of spiked SN}-38\ \text{in blank sample (mg)}} \times 100 \quad (4)$$

Cell culture and animals

The human glioblastoma cell line (U-87MG) was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured and grown in Eagle's Minimum Essential Medium in a humidified atmosphere at 37°C with 5% CO_2. The supplementary solutions were 10% fetal bovine serum, 110 mg/mL of sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Nude mice, 6–8 weeks old with average weight of 18 g from National Laboratory Animal Center (NLAC), Thailand were subcutaneously injected into the right flank with 5×10^6 cells in 50 μL of phosphate buffer saline (PBS). A caliper was used to evaluate tumor volume using the following equation.[29] All procedures were approved by the Institutional Animal Care and Use Committee of the National Animal Center, Thailand.

$$\text{Tumor volume} = (\text{length} \times \text{width}^2)/2$$

Depot administration in tumors

Approximately 21 days after xenograft preparation, animals ($n = 3$ per group) were injected intratumorally by two injections of SN-38 solution or SN-38-loaded depots (60 μL/injection). Injections were continuous every five days for eight times with the same protocol while the non-treatment group was not injected with any depots and solution. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Animal Center, Thailand.

Quantitative analysis of SN-38 in tumors

Before quantitative analysis of SN-38 in tumors, the extraction efficiency of SN-38 from tumors was evaluated by injecting three concentrations of SN-38 into blank tumors. Recovery at low, medium, and high amount of SN-38 (0.1, 1, and 15 mg) tumor samples were prepared and extracted using DMSO. Recovery of these samples was calculated as described above. For quantitative analysis of SN-38 in tumors, tumors containing either drug solution or polymeric depots were placed at room temperature and cut at the center. The first half of tumor was weighed before extraction while the other half was kept at −80°C. After that, polymeric depots inside each tumor section were removed and kept at −80°C and then the tumor was chopped into small pieces. Next, DMSO (10 mL) was added and samples were homogenized for 5 min. The extracted solution was centrifuged at 10°C and 4000 rpm for 5 min and then the supernatant was centrifuged again at 10°C and 14,000 rpm for 10 min. After that, the supernatant was filtered by 0.45 and 0.2 μm nylon filter, respectively, prior to the HPLC analysis. The same procedure was used for the second half of tumor. The polymeric depots were brought out, and then the rest of tumors were extracted and analyzed by HPLC.

Table 3 Summary of QC sample preparation ($n = 30$)

QC sample	Estimated amount of SN-38 (mg)	Actual amount of SN-38 (mg)	% yield[a]
Low	0.11	0.10 ± 0.003	90.90
Medium	1.19	1.07 ± 0.124	89.92
High	15.60	14.67 ± 1.469	94.04

[a]%yield is the actual amount of SN-38 in QC samples (Target SN-38 – Leakage SN-38).

Results and discussion

Preparation of QC samples

QC samples were used to develop extraction and HPLC protocols. In this study, porcine tissues were selected as the extraction model because high number of samples was required in the validation procedures. It should be noted that there was a small SN-38 leakage out of samples during injection. Accordingly, the actual amount of SN-38 inside tissues was determined by subtracting the leakage drug from the amount that was injected. Using this preparation technique, the actual amount of SN-38 inside samples was precisely determined and the extraction efficiency was reliable. The average yields of SN-38 in QC samples after subtracting the leakage amount are shown in Table 3.

Extraction conditions of SN-38 from QC samples

Several methods have been reported for extraction of SN-38 or CPT family in several types of tissues including heart, liver, kidney, lung, spleen, brain, small intestine, and tumor as shown in Table 1. However, most of the published extraction methods for quantification of SN-38 and CPT in tumors did not report the real extraction efficiency from the extraction method.[23,30–34] In these reports, blank homogenates were spiked with drug solution to estimate extraction efficiency which was reported as accuracy of analytical method of instrument.[35,36,38–40] Herein, we determined the extraction efficiency (%) of SN-38 inside tissues and tumors. The actual amount of SN-38 inside each sample could be calculated.

Solvents for SN-38 extraction from QC samples were varied and analyzed. The extraction solvent providing the highest yield was selected to extract SN-38 from tumors. In this experiment, seven types of solvents were used in the extraction which can be divided into two protocols as shown in Table 2. For protocol 1, the extraction was done in two steps. First, hexane, acetone, chloroform–methanol (4:1, v/v), or DMSO–methanol–THF (5:50:45, v/v) were used as solvents and were evaporated out. Next, DMSO

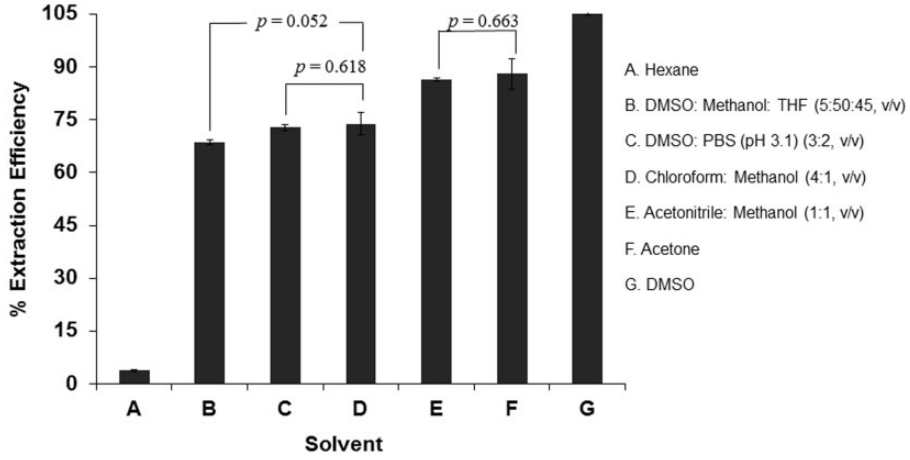

Figure 1 Summary of extraction efficiency (%) using different solvents

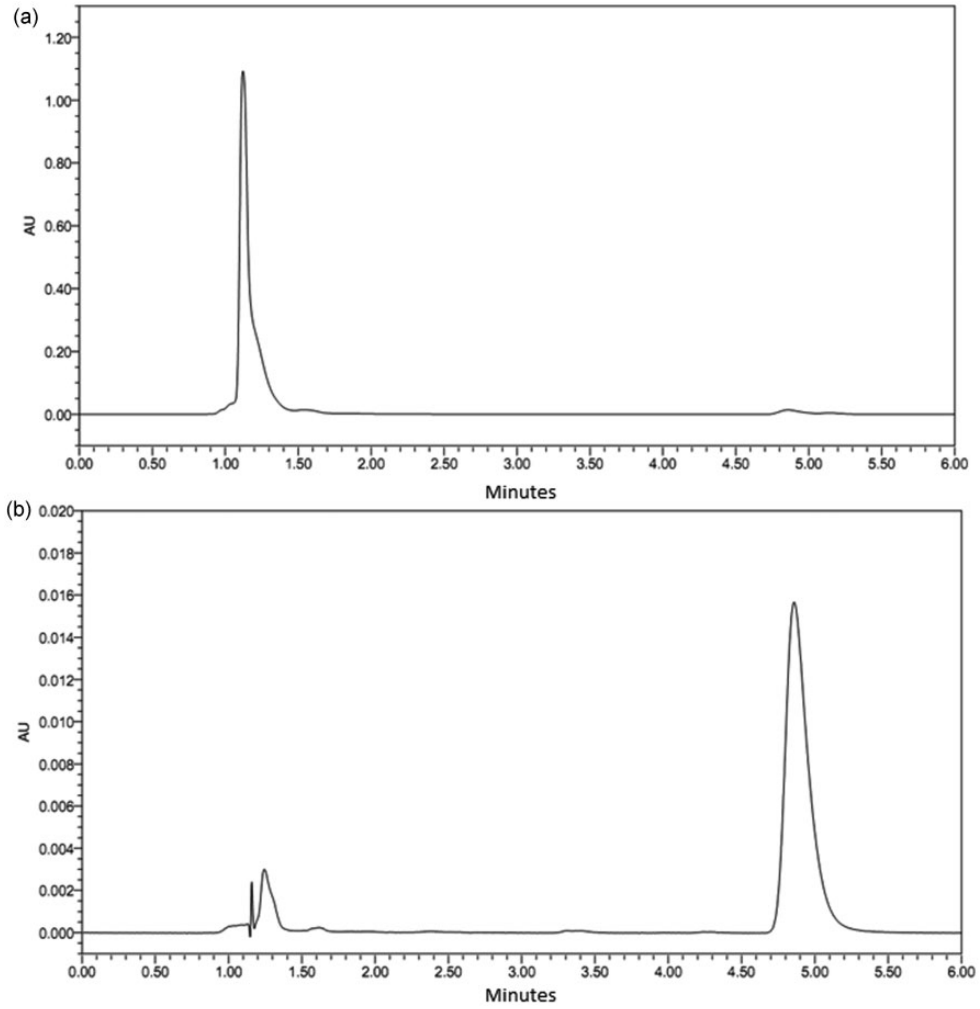

Figure 2 Comparison of chromatograms of SN-38 at concentration of mg/mL using wavelength at (a) 265 nm and (b) 380 nm

was added to dissolve SN-38. For protocol 2, acetonitrile–methanol (1:1, v/v), DMSO–PBS (pH 3.1) (3:2) or DMSO were used as solvents in single step extraction. The extraction efficiency of seven different solvents was evaluated using standard calibration curve of SN-38 in each solvent which were 3.80 ± 0.29, 68.65 ± 0.72, 72.81 ± 0.82, 73.85 ± 3.23, 86.53 ± 0.45, 88.0 ± 4.41, and $105.05 \pm 0.45\%$ for solvent A to G, respectively (Figure 1). Results show

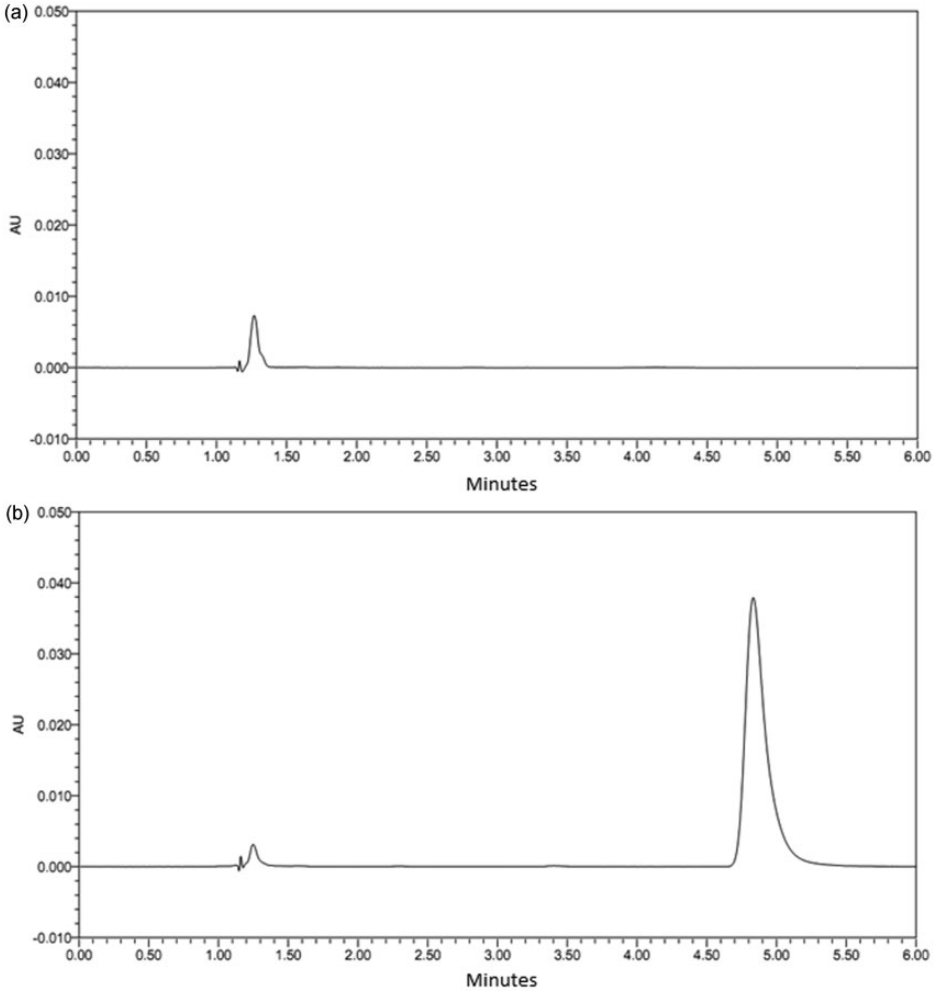

Figure 3 Chromatograms of (a) blank tissues and (b) QC samples at the injection volume of 10 μg/mL

that hexane provided very low extraction efficiency. This was presumably due to hydrophobicity of hexane and as a result it could not penetrate through tissues. The mixture of chloroform and methanol (4:1, v/v) was reported as a solvent for CPT extraction in plasma.[41] DMSO–methanol–THF (5:50:45, v/v) was used to prepare SN-38-loaded-lipid nanocapsules.[42] Nevertheless, these solvents provided lower extraction efficiency of SN-38 from tissues than that of solvent E to F. For solvent C, PBS was mixed with DMSO in order to increase penetration of DMSO into tissues but the yield was still less than 80%. Acetone and the mixture of acetonitrile and methanol provided similar yield, yet still less than using only DMSO. Statistical analyses were performed by sample t-test to compare different extraction techniques with solvent G (DMSO). The result shows that solvent G (DMSO) was statistically significantly different from other extraction techniques ($P < 0.01$). Therefore, DMSO was selected as an extracted solvent to determine the amount of SN-38 in tumors.

Optimization of HPLC conditions

SN-38 can be reversible between active (lactone) and inactive (carboxylate) form under acidic and basic conditions, respectively.[43] In this experiment, the buffer which was $NaH_2PO_4.H_2O$ (pH 3.1, 25 mM) and acetonitrile (70:30, v/v) were used to control the pH of the mobile phase to be in acidic range.[28,43] Two variables were considered and adjusted including injection volume and wavelength for the analysis of SN-38 in tumors. AUC, peak height, retention time of SN-38, and void peak were compared by varying these two parameters. The height of void peak at 1.15–1.25 min was reduced when the wavelength at 380 nm was used as shown in Figure 2. This also resulted in higher peak height of SN-38 at 4.86 min. Peak shape was more symmetrical when the injection volume was reduced from 50 to 10 μL. Finally, the wavelength at 380 nm and injection volume at 10 μL was selected as the best method to provide the best resolution. This method resulted in excellent separation and optimized peak shape of SN-38 without any interference as shown in Figure 3. The retention time of SN-38 was stable at 5.28 min and the total run time was 6.0 min which was less than the previous reports.[43,44–49]

Validation procedures

Selectivity

The selectivity of this method was examined to achieve adequate peak spacing between interference and SN-38 peak. Compared to drug-free tumor samples (blank),

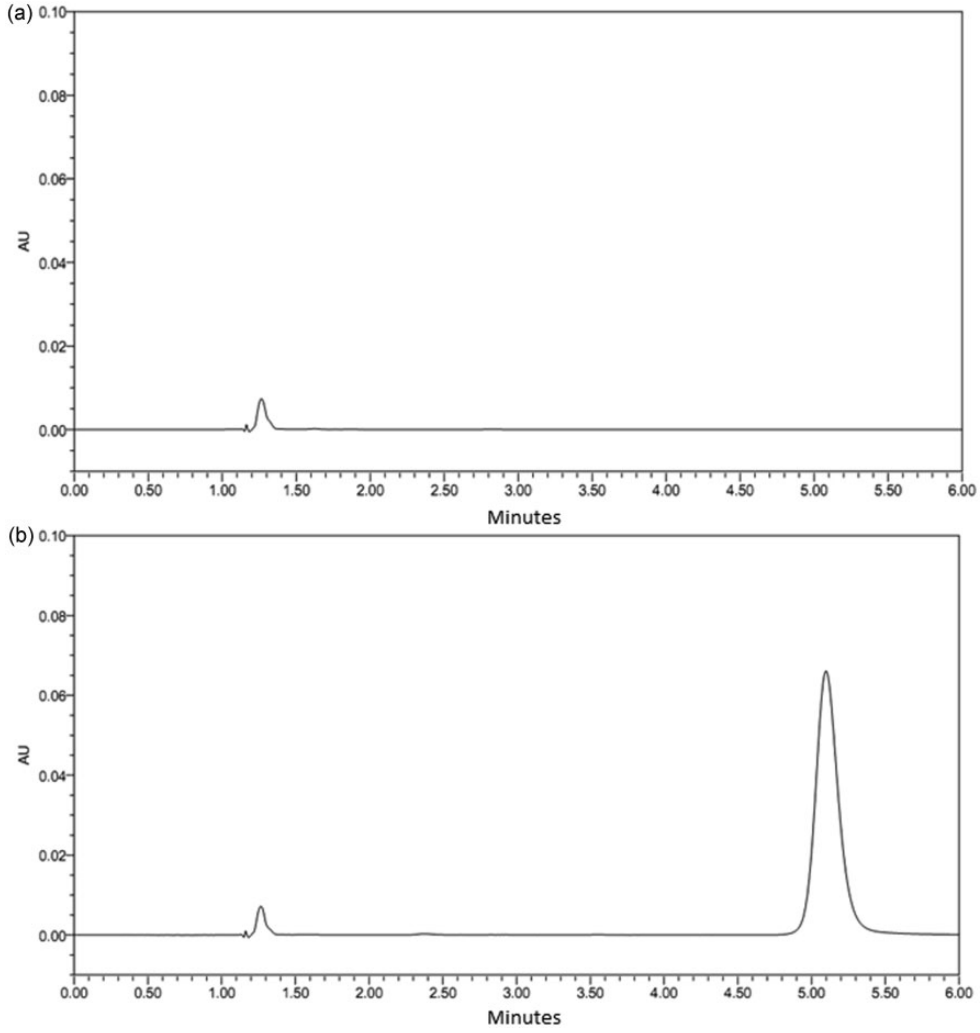

Figure 4 Chromatograms of (a) blank tumors (no depots) and (b) tumors injected by depots

Table 4 Precision and accuracy of extraction procedure determined by HPLC

		Within-day			Between-day		
Concentration range of QC samples	Estimated amount of SN-38 (mg)	Actual amount of SN-38 (mg)	% RSD	% Accuracy	Actual amount of SN-38 (mg)	% RSD	%Accuracy
Low	0.11	0.11 ± 0.002	1.82	100.00	0.10 ± 0.003	3.00	90.91
Medium	1.19	1.41 ± 0.010	0.71	118.49	1.29 ± 0.120	9.30	108.40
High	15.60	16.74 ± 0.320	1.91	107.31	16.33 ± 1.320	8.08	104.67

there was no interference signal in the samples with spiked SN-38. The peak of void was eluted at the beginning (1.16–1.25 min) which was clearly separated from peak of SN-38 at 5.28 min. Both QC samples and tumor samples represented an excellent selectivity as shown in Figures 3 and 4.

Calibration curve, linearity, and sensitivity

Calibration curves were performed by plotting the peak area of SN-38 in DMSO versus its theoretical concentration. The calibration curve presented a linear relationship in the range from 0.03 to 150 μg/mL and provided the correlation coefficient (r^2) more than 0.998 for all analyses. LLOD and LLOQ for this method were 0.308 μg/mL and 1.02 μg/mL, respectively. LLOD was found to be similar to Xuan et al.,[43] whereas LLOD (0.308 μg/mL) was higher than those reported in pharmacokinetic study (20 ng/mL),[45] yet it was low enough to determine the amount of SN-38 in tumors.

Precision and accuracy

The within-day precision and accuracy as well as the between-day precision were very satisfactory and summarized in Table 4. At low concentration, the within-day RSD

Table 5 Average extraction efficiency (% recovery) of porcine and tumor samples

Concentration range of QC samples	Estimated amount of SN-38 (mg)	Extraction efficiency (% recovery)	
		Porcine tissues ($n = 8$)	Tumor ($n = 3$)
Low	0.1	97.60 ± 5.35	117.76 ± 4.52
Medium	1.0	110.09 ± 5.99	91.40 ± 0.03
High	15.0	108.86 ± 3.99	90.95 ± 1.29

was 1.82% and the between-day was 3.00% for all samples. At medium concentration of all analytes, the within-day RSD was 0.71% and the between-day RSD was 9.30%. At high concentrations, the precision were 1.91 and 8.08%, as the within-day and between-day, respectively. The accuracy ranged from 90.91 to 118.49% at known low, medium, and high concentrations of all analyses. The use of internal standard was not necessary due to acceptable linearity of the calibration curve ($r^2 \geq 0.998$), within-day and between-day precision of measurement of QC samples.[28] All validation results show that this HPLC method was ready to apply for quantitative analysis of SN-38 in tumors.

Extraction efficiency of SN-38 from QC samples and tumors using DMSO

SN-38 in QC samples and tumors was prepared at 0.1, 1.0, and 15 mg as the representative value of low, medium, and high amount of SN-38 in tissues. Porcine and tumor samples were homogenized with DMSO which allowed DMSO to dissolve SN-38 out of tissues. Supernatant was separated from residual tissues by two times of centrifugation. For QC samples at medium and high amount of SN-38 (1.0 and 15 mg), only 10 mL of DMSO might not be enough to extract SN-38 from tissues. Thus, sediment of tissues from the first extraction was homogenized again with fresh 10 mL DMSO in order to extract the residual SN-38. The extraction efficiency of SN-38 in porcine and tumor tissues was examined as shown in Table 5. Both porcine and tumor samples provide recovery within 91.40 to 117.76% which indicated the high degree of SN-38 extraction. Due to this small difference of extraction efficiency between porcine and tumor samples, porcine tissues can be used as QC samples instead of tumors. It should be noted that no concentration dependence was observed for SN-38 extraction from both QC samples and tumors.

Quantitative analysis of SN-38 in tumors

Tumors were divided into three groups which were tumors without SN-38 (blank), tumors treated with SN-38 solution, and tumors treated with multiple injections of SN-38-loaded polymeric depots. These tumors were extracted by DMSO (Protocol G) and analyzed by HPLC as described in previous sections. Chromatograms of tumors with and without SN-38 are shown in Figure 4. It should be noted that tumors without SN-38 (the control group) were extracted which resulted in smooth baseline and no interferance within 6 min. Results show that tumors treated with SN-38 solution contained lower amount of SN-38 remaining in tumor tissues than those treated with SN-38-loaded polymeric depots where the remaining percentages of SN-38 in tumors were 17.73 ± 11.40 and 38.46 ± 7.16, respectively ($P < 0.01$) (Table 6). This result shows that SN-38-loaded polymeric depots prevented the leakage of free-drug from tumors and sustained higher amount of SN-38 inside tumors by gradual release of SN-38 from polymeric depots. Thus, the therapeutic efficacy can be elivated using SN-38-loaded polymeric depots.

Table 6 Determination the amount of SN-38 in tumors ($n = 3$.)

SN-38 injection	Remaining SN-38 in tumors (mg)	Remaining SN-38 in tumors (%)
SN-38 solution	6.05 ± 0.69	17.73
SN-38 loaded-polymeric depots	13.83 ± 0.99*	38.46

*$P < 0.01$, versus SN-38 solution.

Conclusion

Extraction and HPLC analysis of SN-38 were optimized and validated. Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were 0.308 μg/mL and 1.02 μg/mL, respectively. The extraction efficiency (%recovery) of SN-38 in porcine tissues was similar to that of tumors which provided more than 90% recovery in all concentrations. Moreover, the variability of precision and accuracy within and between-day were less than 15%. The method was simple, quick, reliable, and has been successfully applied to determine the amount of SN-38 in tumors after administrated by SN-38 solution and SN-38-loaded polymeric depots. Results show that intratumoral injection of SN-38-loaded polymeric depots produced higher and sustainable SN-38 release in tumors which could elevate therapeutic efficacy of SN-38.

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