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Short communication

HPLC method for determination of SN-38 content and SN-38 entrapment efficiency in a novel liposome-based formulation, LE-SN38

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Abstract

A simple HPLC method was developed for quantification of SN-38, 7-ethyl-10-hydroxycamptothecin, in a novel liposome-based formulation (LE-SN38). The chromatographic separation was achieved on an Agilent Zorbax SB-C18 (4.6 mm × 250 mm, 5 μm) analytical column using a mobile phase consisting of a mixture of NaH_2PO_4 (pH 3.1, 25 mM) and acetonitrile (50:50, v/v). SN-38 was detected at UV wavelength of 265 nm and quantitatively determined using an external calibration method. The limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.05 and 0.25 μg/mL, respectively. The individual spike recovery of SN-38 ranged from 100 to 101%. The percent of relative standard deviation (%R.S.D.) of intra-day and inter-day analyses were less than 1.6%. The method validation results confirmed that the method is specific, linear, accurate, precise, robust and sensitive for its intended use. The current method was successfully applied to the determination of SN-38 content and drug entrapment efficiency in liposome-based formulation, LE-SN38 during early stage formulation development.
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Keywords: HPLC; SN-38; Liposomes; Validation; Entrapment efficiency

1. Introduction

SN-38 (7-ethyl-10-hydroxycamptothecin) is an active metabolite of irinotecan (CPT-11), a derivative of camptothecin that is commercially available as Camptosar® for Injection for the treatment of recurrent, metastatic colorectal cancer (Fig. 1). The metabolic conversion of CPT-11 to the active SN-38 occurs in the liver via carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and a dipiperidino side chain [1]. It is reported that only a small fraction (2–8%) of CPT-11 administered into the human body is converted to SN-38 [2], hence a large dose of CPT-11 is needed to achieve therapeutic effectiveness. In addition, the conversion of CPT-11 (micromolar plasma levels) to SN-38 (nanomolar plasma levels) is highly variable from patient to patient and thus poses significant life threatening toxicity risks, and complicates the clinical management of patients. SN-38, on the other hand, has an advantage over its camptothecin precursors in that it does not require activation by the liver, thereby greatly reducing the interpatient variability. Additionally, SN-38 is approximately 1000-fold more potent than CPT-11 as a topoisomerase I inhibitor against several tumor cell lines [4,1–3]. However, the development of this drug has been largely hindered by the poor solubility and stability of SN-38 in pharmaceutically acceptable solvents. A novel and well-characterized liposome-based SN-38 formulation (LE-SN38) was developed that would overcome the aforementioned problems, and provide a mechanism of direct delivery of SN-38 without in vivo enzymatic conversion. In pre-clinical studies, LE-SN38 exhibited better efficacy against various mouse tumor xenograft models as compared to CPT-11 at similar dose levels [5].

To support ongoing formulation development and other pre-clinical studies, a specific and sensitive analytical method was needed. The objective of this study was to develop a simple HPLC/UV method for determination of SN-38 content and SN-38 entrapment efficiency in liposome-based formulation to support early stage formulation development.

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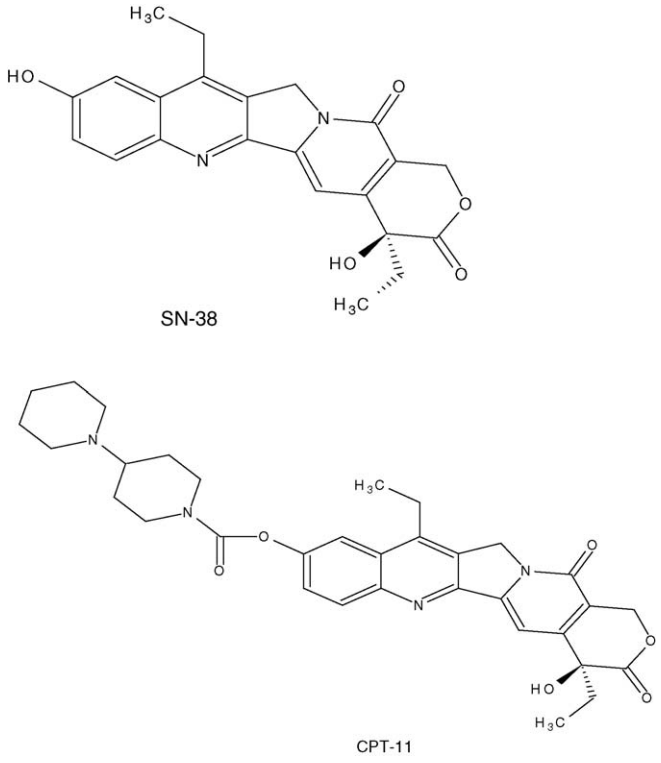


Fig. 1. Structure of SN-38 and CPT-11.

2. Experimental

2.1. Chemicals and reagents

SN-38 was purchased from Qventas (Newark, DE). Acetonitrile, 85% phosphoric acid and sodium phosphate monobasic were purchased from Fisher Scientific (Pittsburgh, PA). Purified water was obtained from Milli-Q water system (Millipore Corp, Bedford, MA). Sodium hydroxide (5 M) was purchased from LabChem, Inc. (Pittsburgh, PA). Hydrogen peroxide (30%) was purchased from VWR Scientific (West Chester, PA). All chemicals were used as received without further purification.

2.2. Chromatographic system and conditions

The HPLC system consists of an Agilent 1100 module (Wilmington, DE), including quaternary pump, mobile phase degasser, auto-sampler with thermostat, column oven, coupled with a multiple wavelength or diode array UV detector. Agilent Zorbax SB-C18 (4.6 mm × 250 mm, 5 μm) and Phenomenex Nucleosil C18 (4.0 mm × 250 mm, 5 μm) analytical columns were used for method development and validation. The ChemStation chromatographic software was used for data acquisition and processing. The mobile phase consisted of a mixture of NaH_2PO_4 (pH 3.1, 25 mM) and acetonitrile (50:50, v/v%). During assay, an aliquot of 20 μL of diluted sample of liposome-based SN-38 was injected in duplicate into the analytical column at 40 °C at a flow rate of 1 mL/min. SN-38 was detected at 265 nm and quantitatively determined using an external calibration method. The analytical column theoretical plate number and tailing factor of the analytes under different chromatographic conditions were calculated using USP methods [6].

2.3. HPLC method development

2.3.1. HPLC conditions

Different mobile phase conditions, such as, compositions, buffer strength, buffer pH and column temperature were tested to improve the reproducibility and peak shape, and also to ensure the lipid components and other excipients in the formulation are completely soluble.

2.3.2. Lactone-carboxylate forms conversion

The lactone ring of SN-38 is unstable and converts to an open-ring carboxylate form under basic conditions. A study of lactone-carboxylate structural conversion of SN-38 under the different pH conditions was carried out to determine the optimal media pH in which the structural conversion was minimal. In the study, SN-38 samples were prepared separately by diluting the SN-38 stock solution with water and acetonitrile mixture (water/acetonitrile = 50:50, v/v) at various pH conditions, and subsequently analyzed by HPLC.

2.4. Liposome-based SN-38 (LE-SN38) preparation

LE-SN38 was prepared as previously described [5]. Briefly, phosphatidylcholine, cholesterol, cardiolipin and α-tocopherol were dissolved in ethanol and transferred into a suitable round bottom flask. The flask was then connected to a Büchi R205 rotary evaporator (Flawil, Switzerland) and water bath (Buchi B-490) to remove ethanol until a lipid film was formed on the flask wall. The lipid film was then hydrated with SN-38 and sucrose solution to form liposomes. The performed liposomes were size reduced by extrusion under pressure through two stacked of 0.1 μm polycarbonate filters (Whatman, Inc., Clifton, NJ, USA). The mean vesicle size of the final liposomes was reduced to about 150 nm. The resulting liposomes were then sterile-filtered through 0.2 μm filter and filled into 10-mL serum vials prior to be lyophilized in VirTis lyophilizer (Genesis, The VirTis Company, Gardiner, NY, USA).

2.5. Drug entrapment efficiency

Drug entrapment efficiency (EE) was determined by both ultra-centrifugation and dialysis methods. For ultra-centrifugation method, an aliquot of the reconstituted LE-SN38 was diluted four-fold with normal saline and then centrifuged at 200,000 × *g* for 2 h at 4 °C using a Beckman Optima™ Series L-90 K ultracentrifuge (Palo Alto, CA, USA) with a SW 60 Ti rotor. The total drug concentrations in liposomes before centrifugation and in supernatant after centrifugation were determined by the current HPLC method. The percentage of the drug entrapped in the liposomes was calculated as follows:

$$\text{drug entrapment}\,(\%)\frac{\text{drug}_{\text{total}} - \text{drug}_{\text{supernatant}}}{\text{drug}_{\text{total}}} \times 100$$

Dialysis of SN-38 from LE-SN38 was conducted by membrane dialysis against phosphate-buffered saline (PBS, pH 7.4) at 37 °C. Briefly, a 2 ml aliquot of reconstituted LE-SN38 sample was placed in the dialysis cassette (Pierce Slide-A-Lyzer® dialysis cassettes with a MW cutoff of 10 K) and then suspended in a temperature-controlled, jacketed flask containing 400 mL of PBS. At various time intervals, aliquot samples were withdrawn and subjected to SN-38 content analysis by the current HPLC method.

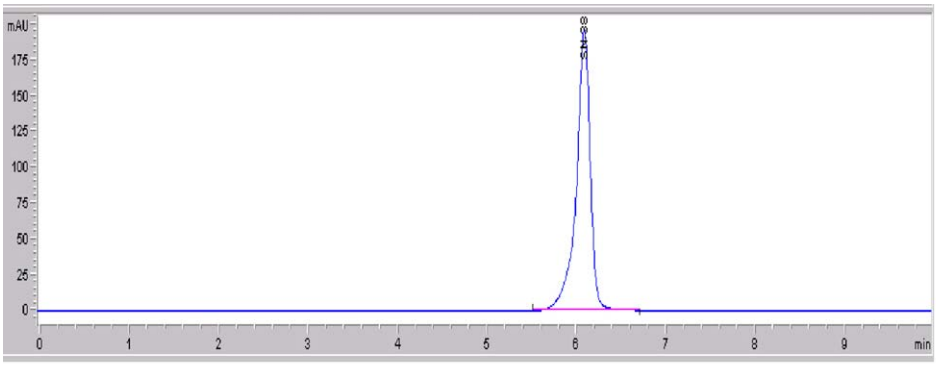
3. Results and discussion

3.1. Method development

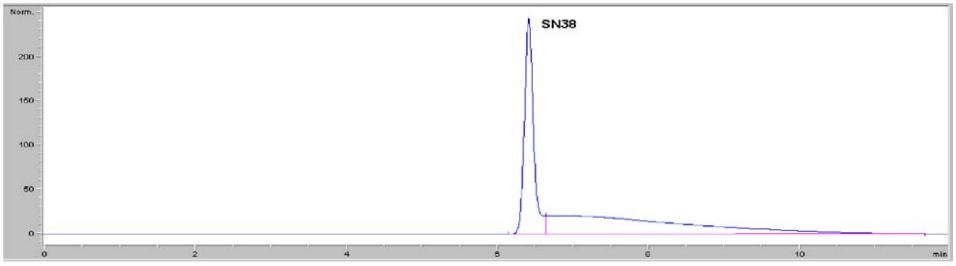
3.1.1. HPLC conditions

The chromatographic behavior of SN-38 was first studied using the method reported by Escoriaza et al. [7]. In this study, the SN-38 standard (30 μg/mL) was analyzed on a Nucleosil C18 (Phenomenex, 4.0 mm × 250 mm, 5 μm) analytical column at 30 °C using NaH_2PO_4 (pH 4, 100 mM) and acetonitrile (67:33, v/v) as a mobile phase at a flow rate of 1 mL/min. Zorbax SB-C18 analytical column (Agilent, 4.6 mm × 250 mm, 5 μm) was also evaluated under the same chromatographic conditions. It was found that retention time of SN-38 shifted as much as 3% among five replicate injections when the Nucleosil C18 was used. Severe peak fronting (tailing = 0.78) was also noticed (Fig. 2a). When the Zorbax SB-C18 column was used, a shift as much as 8% in retention time and severe peak tailing were observed (Fig. 2b). The shift in retention time and peak distortion of SN-38 observed under these conditions were most likely caused by a combination of the high phosphate buffer concentration in the mobile phase and low column temperature. In order to prevent the retention time shift during analysis and improve the peak shape of SN-38, the effects of the column temperature and buffer strength were further investigated. It was found that by increasing column temperature to 40 °C and reducing phosphate buffer concentration from 100 mM to about 25 mM, the shift in retention time was minimal and the SN-38 peak shape was significantly improved. Taking into consideration of the low solubility of lipid components of LE-SN38 in aqueous media, the ratio of organic to aqueous phase in the mobile phase was increased from 37/63 to 50/50 v/v. Consequently a mixture of NaH_2PO_4 (pH 3.1, 25 mM) and acetonitrile (50:50, v/v%) was chosen as mobile phase.

With the above changes, the chromatographic behavior of SN-38 was re-evaluated using both Zorbax SB-C18 and Nucleosil C18 columns at 40 °C. It was found that there were no retention time shifts for either of these analytical columns, and no peak distortion for Zorbax SB-C18 column under the new condition. However, peak fronting was still noticed when Nucleosil C18 column was used, Moreover, the Nucleosil C18 column caused higher HPLC system pressure (34% higher) as compared to that when the Zorbax SB-C18 column was used. Consequently, Zorbax SB-C18 was selected as the primary column for method development and remained as the column utilized in the method validation work.



(a)



(b)

Fig. 2. Chromatograms of SN-38 under different HPLC conditions: (a) mobile phase was a mixture of NaH_2PO_4 (pH 4, 100 mM)/acetonitrile (67:33, v/v), flow rate at 1 mL/min, Nucleosil C18 column (4.0 mm × 250 mm, 5 μm), column temperature at 30 °C, UV wavelength = 265 nm; (b) same as (a) except the column was a Zorbax SB-C18 (4.6 mm × 250 mm, 5 μm).

3.1.2. Lactone-carboxylate forms conversion

The carboxylate form and lactone form of SN-38 were baseline separated ($R = 4.9$) under the new chromatographic conditions. It was found that about 60% of SN-38 existed in carboxylate form and 40% in closed lactone ring structure when pH of the diluent was kept in neutral (pH 7.09). When pH of the diluent was raised to 10.2, roughly 95% of the SN-38 was in carboxylate form and only 5% was in lactone ring form. Once pH of the diluent was adjusted to 3.4, almost all the open carboxylate form of SN-38 converted to lactone ring structure (Fig. 3). This pH-dependent structural conversion was found to be reversible; therefore, it is imperative that the pH of the sample diluent and mobile phase be kept at the same level to prevent structural conversion during sample preparation and assay.

3.2. Method validation

3.2.1. Specificity

To assure the specificity of the current method, the liposome formulation placebo containing the same concentration of lipids and other excipients as in the active formulation was evaluated. Forced-degradation experiments were also preformed on the SN-38 samples under different stressed conditions. The stress conditions used were thermal (60 °C for 2 h), acid (0.1 M HCl, 20 h in the dark at room temperature) and base (0.1 M NaOH, 20 h in the dark at room temperature) treatments and oxidation (3% H_2O_2, 20 h in the dark at room temperature). The percentage of degradation under each stressed condition was calculated based on the recovery of SN-38 in the sample after being stressed.

Peak purity analysis using a photodiode array detector was performed to confirm that the response was not caused by more than one component. The experiment results showed that the lipid components and other excipients in the formulation had no interference with SN-38 peak. The results of the forced degradation study are summarized in Table 1, and chromatograms for samples degraded under different stress conditions are shown in Fig. 4. As expected, SN-38 completely converted to the carboxylate form under 0.1 M NaOH condition (Fig. 4a). The carboxylate and lactone ring forms of SN-38 were chromatographically baseline separated under the HPLC conditions. Under acidic condition (0.1 M HCl), SN-38 degraded about 11% and generated an unknown degradant. The degradant and SN-38, however, were baseline resolved ($R = 6.6$, Fig. 4b). About 15% degradation was noticed when SN-38 was treated with 3% H_2O_2 (Fig. 4c). The oxidative degradant was baseline separated from SN-38 ($R = 5.8$). Under thermal treatment at 60 °C for 2 h, no degradation of SN-38 was discovered suggesting that SN-38 was relatively heat stable.

Table 1
Results of SN-38 forced degradation study

Treatment	% Degradation	Results
0.1 M NaOH	100	Completely converted to carboxylate form
0.1 M HCl	11	Deg. peak baseline sep. from SN-38 ($R = 6.6$)
3% H_2O_2	15	Deg. peak baseline sep. from SN-38 ($R = 5.8$)
Heated at 60 °C	0	No degradation

3.2.2. Linearity

The relationship between SN-38 concentration and detector response was evaluated to confirm linearity. SN-38 standards at five different concentrations encompassing minimum of 80–120% of the expected concentration of SN-38 in LE-SN38 were included in the study. It was found that in the concentration range of 1–25 μg/mL, the detector response of SN-38 was linear with a correlation coefficient greater than 0.999.

3.2.3. Accuracy (spike recovery)

SN-38 spike samples were prepared by the addition of SN-38 standard solution to the liposome placebos at 80%, 100%

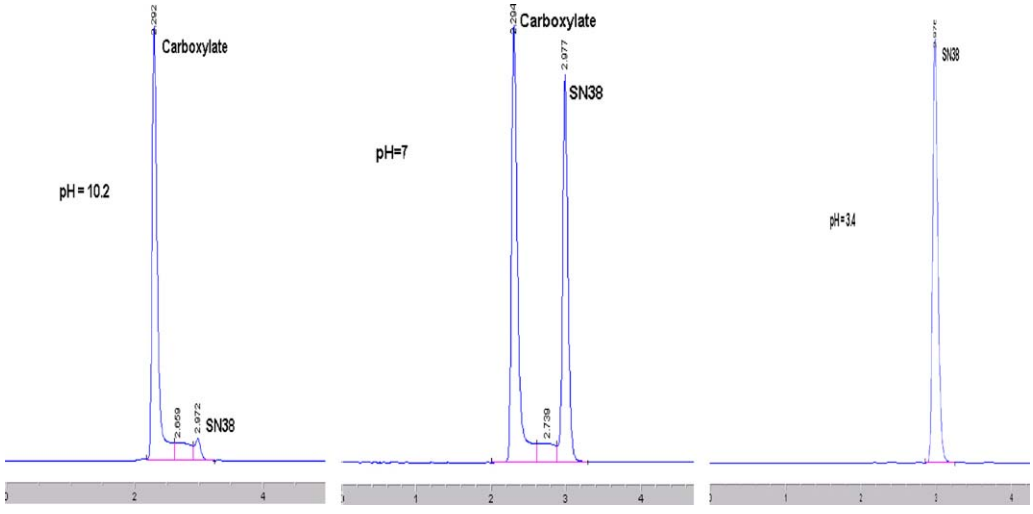
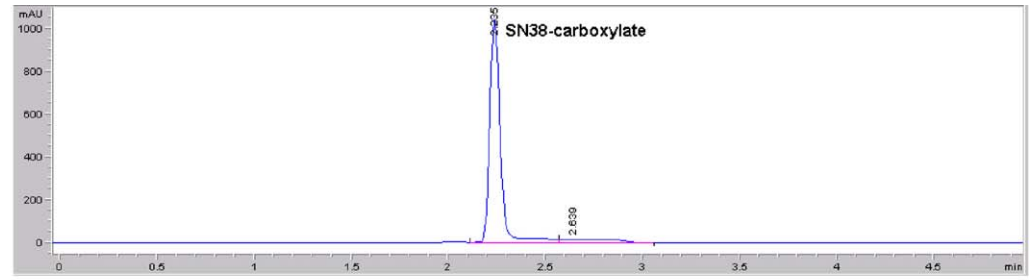
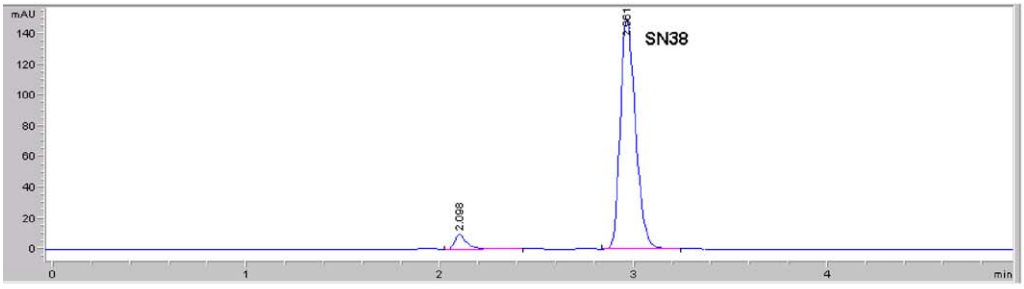


Fig. 3. Ratio of lactone and carboxylate forms of SN-38 at different pH levels.



(a)



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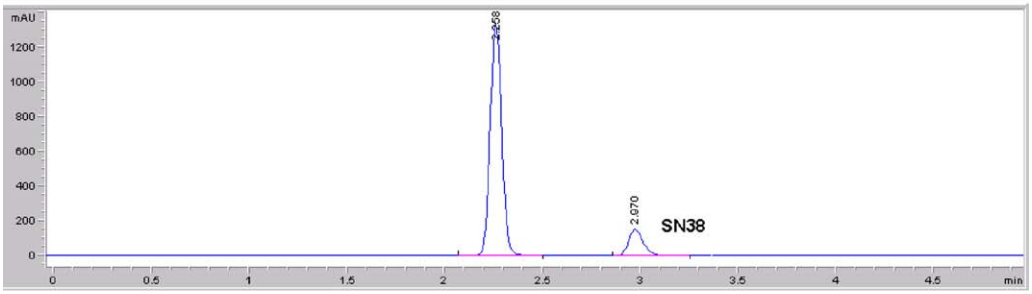

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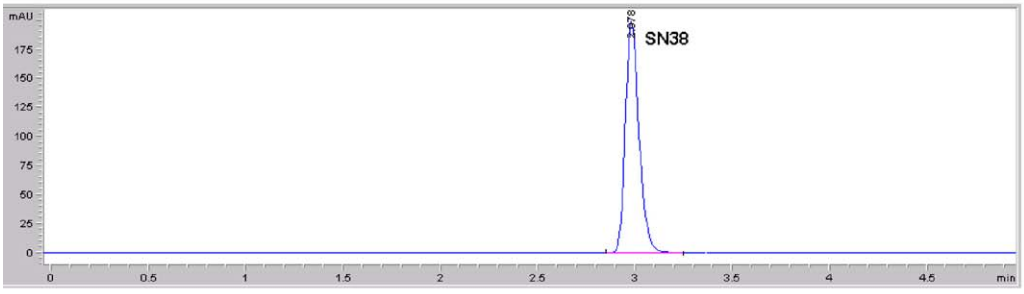

(d)

Fig. 4. Chromatograms of SN-38 specificity samples: (a) treated with 0.1 M NaOH (20 h); (b) treated with 0.1 M HCl (20 h); (c) treated with 3% H_2O_2 (20 h); (d) heated at 58 °C for 2 h.

and 120% of the expected concentration of SN-38 in LE-SN38. The average percent recoveries of SN-38 in the spiked samples at three different levels ranged from 99.9% to 100.7% with an overall average of 100.3%. The percent of relative standard deviation (%R.S.D.) for the triplicate measurements at each level was less than 0.5% (Table 2), which demonstrated a high level of accuracy.

3.2.4. Precision

The precision of the current method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). The repeatability was done by analyzing six aliquots of LE-SN38 sample from a single container (triplicate injections of each). The same process was repeated on a second day to assess intermediate precision using six freshly prepared aliquots of LE-SN38

Table 2
Results of accuracy (spike recovery) study

Sample	Recovery (%)	Average recovery (%)	%R.S.D.
SN-38 spike at 80% level-1	100.1	100.3	0.2
SN-38 spike at 80% level-2	100.5		
SN-38 spike at 80% level-3	100.3		
SN-38 spike at 100% level-1	100.7	100.7	0.06
SN-38 spike at 100% level-2	100.8		
SN-38 spike at 100% level-3	100.7		
SN-38 spike at 120% level-1	99.7	99.9	0.21
SN-38 spike at 120% level-2	100.1		
SN-38 spike at 120% level-3	99.8		

Table 3
Results of precision and intermediate precision study

Sample	%R.S.D., day-1 (triple injections)	%R.S.D., day-2 (triple injections)
Assay-1	0.16	0.07
Assay-2	0.09	0.07
Assay-3	0.19	0.00
Assay-4	0.07	0.06
Assay-5	0.06	0.13
Assay-6	0.07	0.14
Overall %R.S.D. (18 injections)	1.51	0.79

sample from the same container and the same SN-38 standards. The precision was measured by the %R.S.D. of the triplicate injections for each of the six samples. It was found that the precision was 1.51% on day-1 and 0.79% on day-2 (Table 3). These data demonstrated the acceptable precision of the method.

3.2.5. Robustness

The robustness of the current method was assessed by analyzing HPLC system suitability using a SN-38 standard (25 μg/mL, five replicate injections) under deliberately altered conditions including changes in column temperature, organic-to-aqueous ratio, and pH in mobile phase and mobile phase flow rate. The resulting retention time and system suitability parameters, such as theoretical plate number, detector response (peak area) and tailing factor under each changed condition are shown in Table 4.

When the organic-to-aqueous ratio of the mobile phase was decreased or increased by 2%, the corresponding retention time increased or decreased by 3.6%, and the theoretical plate number increased by up to 1.5%, whereas the detector response and tailing factor remained unchanged. Changes in column temperature by ±2 °C had no impact on the retention time, detector response and tailing factor of SN-38; however, the theoretical plate number increased by 4% (Table 4). While changes in pH of the mobile phase (±0.2 unit) had no influence on SN-38 retention time, detector response and tailing factor, the theoretical plate number increased by up to 2.4% (Table 4).

Mobile phase flow rate had expected effects on SN-38 retention time, theoretical plate number and detector response. As the mobile phase flow rate was decreased to 0.9 mL/min, the SN-38 retention time, theoretical plate number and detector response increased by 15%. When the flow rate was increased to 1.1 mL/min, the SN-38 retention time, theoretical plate number and detector response decreased by 9.2%, 1.9% and 8.9%, respectively. It was observed that changing the flow rate from 0.9 to 1.1 mL/min had little effect on the tailing factor. Overall, under various deliberately altered HPLC conditions, the method demonstrated sufficient ruggedness and is considered acceptable for the analysis of SN-38.

3.2.6. LOD and LOQ determination

The limit of detection (LOD) was established by analyzing SN-38 standards in six replicate injections at different concentration levels in decreasing order until signal to noise (S/N) ratio reached about 3. The limit of quantitation (LOQ) LOQ was established in the same way as for the LOD, except that the S/N ratio was about 10. The LOD and LOQ of the current method were determined to be 0.05 μg/ml (%R.S.D. = 7.4), and 0.25 μg/ml (%R.S.D. = 1.3), respectively, indicating the method was sufficiently sensitive to be used for the drug entrapment study.

Table 4
Influence of changes in experimental parameters on the performance of chromatographic system

Parameter	Modification	Retention time (minute)	Peak area (% change)	Tailing factor	Theoretical plate number (% change)
Mobile phase ratio (v/v) acetonitrile:buffer	48:52	3.03	0.11	1.1	1.53
	50:48	2.93	n/a	1.1	n/a
	52:48	2.82	0.32	1.0	0.52
pH	2.9	2.93	0.27	1.1	0.83
	3.1	2.93	n/a	1.1	n/a
	3.2	2.93	0.32	1.1	2.42
Flow rate (ml/min)	0.9	3.25	11.4	1.1	15.1
	1.0	2.93	n/a	1.1	n/a
	1.1	2.66	−8.9	1.1	−1.89
Temperature (°C)	38	2.93	0.27	1.1	0.82
	40	2.93	n/a	1.1	n/a
	42	2.93	0.05	1.1	4.09

n/a: not applicable. The results were reported as an average of five replicate injections.

Table 5
SN-38 concentration and entrapment efficiency in different LE-SN38 batches

Batch	SN-38 (mg/mL)	Percentage of the target concentration	% Entrapment[a]	% Entrapment[b]
1[c]	1.74	87.0	99	NA
2[c]	1.62	81.0	N.A	98.1[d]
3[e]	1.87	93.5	100	NA
4[e]	1.93	96.5	100	NA

NA: not available.
[a] Determined by Ultra-centrifugation method.
[b] Determined by dialysis method.
[c] Prepared by thin film hydration procedure.
[d] 1.9% SN-38 released from LE-SN38 after 120 h of dialysis.
[e] Prepared by Ethanol injection procedure.

3.3. LE-SN38 development and drug entrapment

The current method was applied to determine SN-38 content and drug entrapment efficiency (EE) in liposomes. For SN-38 content analysis, LE-SN38 samples were diluted 100-fold with mobile phase, whereas for EE assay, samples were injected directly without dilution due to the expected low concentration of SN-38. Table 5 shows the analytical results for SN-38 content and drug EE in various batches of LE-SN38 during early phase of formulation development.

The SN-38 content was found to be about 81–87% of the target concentration (2 mg/mL) in the prototype formulations (entries 1 and 2, Table 5) due to loss of drug in the formulation process. Subsequently, the process was improved, and the drug contents of the resulting batches were in the range of 94–97% of the target value (entries 3 and 4, Table 5).

The drug EE, determined by ultracentrifuge method, was found to be greater than 99%, which suggests that SN-38 was highly entrapped inside the liposomes. This result was also confirmed by a separate in vitro dialysis study, in which only 1.9% of SN-38 released from LE-SN38.

4. Conclusion

A simple HPLC method was developed and validated for determination of SN-38 content in the novel, liposome-based formulations. Even though SN-38 was formulated in a complex phospholipids matrix, the current method involved no sample pre-treatment or extraction procedures. Only a simple dilution of the formulation prior to HPLC analysis was required. The method validation results indicate that the method is specific, linear, accurate, precise, robust and sensitive that is suitable for determination of SN-38 concentration and drug entrapment efficiency in liposomes to support formulation development and other pre-clinical studies.

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