Development and validation of an ion-pair HPLC chromatography for simultaneous determination of lactone and carboxylate forms of SN-38 in nanoparticles

Development and Validation of an Ion-pair HPLC Chromatography for Simultaneous Determination of Lactone and Carboxylate Forms of SN-38 in Nanoparticles

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ABSTRACT

A simple and reproducible reversed-phase ion-pair high performance liquid chromatography (HPLC) method using isocratic elution with UV absorbance detection was developed and validated for the simultaneous determination of carboxylate (C) and lactone (L) forms of SN-38 (active metabolite of irinotecan) in nanoparticles (NPs). Reversed phase (RP) chromatography was performed on a C18 column with an ion-pair solution, KH_2PO_4 buffer solution and acetonitrile, under a mobile phase 30:30:40 (v/v) and at a flow rate of 1 mL/min. Detection was performed at 265 nm and sharp peaks were obtained for C and L forms of SN-38 at retention times of 2.9 ± 0.01 and 5.1 ± 0.01 min respectively. Linear regression analysis data for the calibration plot showed a good linear relationship between response and concentration in the range of 4 to 100 µg/mL; the regression coefficient was 0.9999 for L form and 0.9998 for C form. The optimized method showed good performance in terms of specificity, linearity, detection and quantitation limits, precision and accuracy in accordance with the International Conference on Harmonization (ICH) Q2 (R1) guidelines. This assay was demonstrated to be applicable for routine quantitation of SN-38 in PLGA NPs.

Key words: SN-38, irinotecan, nanoparticles, ion-pair, HPLC

INTRODUCTION

Camptothecin (CPT) is a natural, water-insoluble quinoline alkaloid produced by an Asian tree, *Camptotheca acuminata* [native to China and Tibet][1]. Camptothecin has been modified to create a host of other anti-cancer drugs, including irinotecan (CPT-11). SN-38 (7-ethyl-10-hydroxycamptothecin), the active metabolite of irinotecan, is 100-1000 folds more cytotoxic than irinotecan against many tumor cell lines. The schematic drawing of SN-38, irinotecan and CPT is shown in Figure 1[1-3]. Only a small proportion (2-8%) of irinotecan is converted to SN-38[2,3], and so a large dosage of irinotecan is needed to obtain therapeutic efficacy. Furthermore, the conversion of irinotecan to SN-38 is extremely variable and thus

causes toxicity risks for the patients[4,5]. Nevertheless, the delivery of SN-38 is wholly challenging due to instability and low water solubility [6,7]. Nanoparticle (NP) technology may provide a solution for such problems and promote a sustained chemotherapy. Drugs incorporated into NPs have also shown increased activity[8]. In our previous study a novel and well-characterized poly lactide-co-glycolide (PLGA) NPs loaded SN-38 was developed to overcome these problems and provided a mechanism of direct delivery of SN-38 without *in vivo* enzymetic conversion[3]. Camptothecins and related analogues contain a closed -hydroxy--lactone ring (L form) that can undergo reversible hydrolysis to form the open-ring form (C form). *In vitro* and *in vivo* pharmacological study showed that the hydroxy acid is a less potent inhibitor of topoisomerase I and a much less potent antitumor agent with much stronger toxic effects[9,10]. Chemical structures and equilibrium reaction between the L and C forms of SN-38

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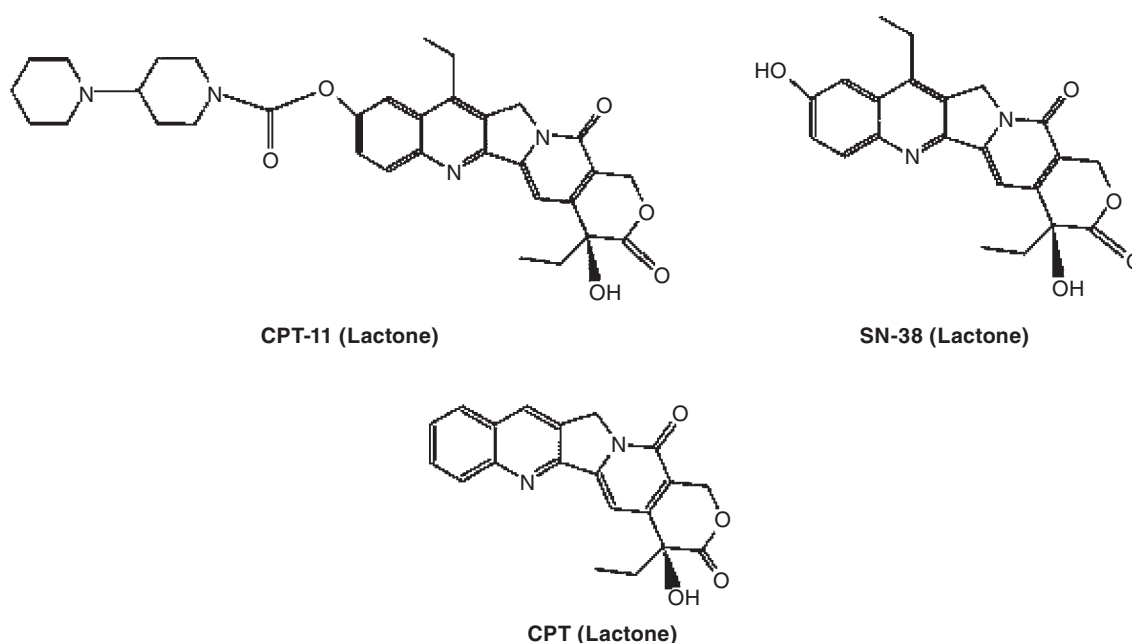
Figure 1. Schematic structure of SN-38, irinotecan and CPT.
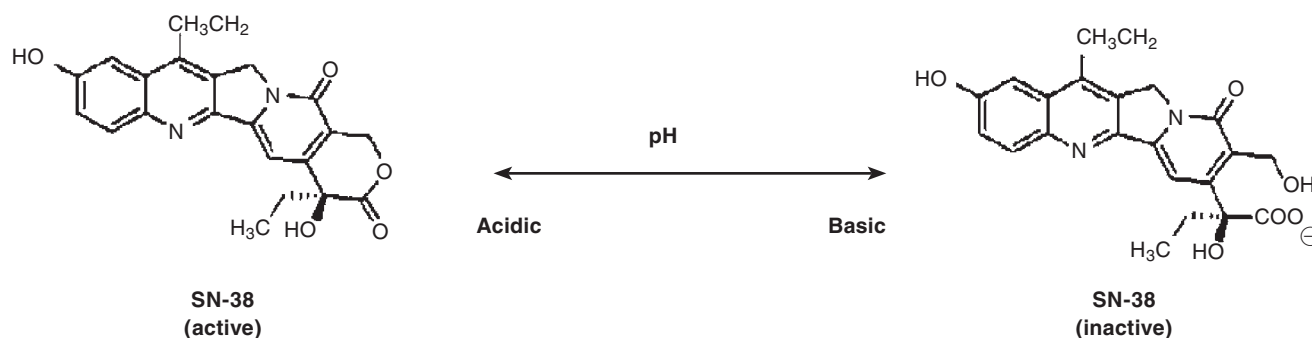
Figure 2. Schematic structure of lactone and carboxylate forms of SN-38.

are shown in Figure 2. Analytical procedures determining camptothecin derivatives could be classified in two groups: methods quantifying total concentration of these drugs and methods that discriminate between the L and C forms. Since L is the active form of these anticancer agents, discriminating between L and C forms appears to be an aim to evaluate the pharmacokinetics and pharmacodynamics of these compounds and in pharmaceutical development[11,12]. The method presented here identifies the L and C forms of SN-38 and determines concentration of SN-38 in NPs. Several methods have been published for the determination of C and L forms of SN-38 in several matrix (plasma, microsomal extracts, and liposomes, etc.), including liquid chromatography-tandem mass spectrometry (LC-MS/MS), high performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescent detector[13-19]. However, no analytical method has been validated for determination of SN-38 loaded PLGA NPs.

To date, most techniques were limited to determine the total concentrations of two forms of SN-38 by transferring carboxylate forms to lactone forms through acidification of the sample[16] or by transferring lactone forms to carboxylate forms through basification of the sample[18]. Some methods were able to determine the carboxylate forms of CPT-11 and SN-38 in plasma by subtracting lactone form from the total form with appropriate process to stop the conversion of lactone form to carboxylate form[16]. However, these methods needed several different HPLC conditions for each sample to determine the lactone form and total form separately. Some methods could separate lactone and carboxylate forms simultaneously by gradient elution[17,21]. Although these methods had similar limit of detection (LOD) or limit of quantitation (LOQ), or similar retention time in comparison with our method, most of them used fluorescence detector[15-19]. We used UV detector in our method

because it is more common than fluorescence detector.

As analysis of SN-38 in NPs is necessary to pass *in vitro* or *in vivo* assay test, valuation of a new formulation of the SN-38 like measuring encapsulation efficiency, or release of particulate drug delivery systems containing SN-38, a simple, reproducible and cost-effective analysis method is important. Additionally, proper sample handling should be adopted to avoid the conversion between these two forms.

The development of this chromatographic method suitable to the quality control of NP formulation is a significant improvement in terms of simple steps and sensitivity. The aim of the presented work was to introduce a simple, sensitive, compendial and rapid isocratic HPLC method for the simultaneous determination of the L and C forms of SN-38 loaded PLGA NPs and to continue formulation development *in vitro* and *in vivo*.

MATERIALS AND METHODS

I. *Materials*

SN-38 was obtained from ABATRA Co., Shaanxi, China. PLGA (Resomer 504) was purchased from Boehringer Ingelheim, Germany. Poly vinyl alcohol (PVA) (MW 22,000) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The ion-pairing reagent octane - 1- sulfonic acid sodium salt was purchased from Sigma Chemical (St. Louis, MO, USA). Dichloromethane (DCM) (analytical grade) and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). The Purified water was prepared using Milli-Q water system (Millipore Corp, Bedford, MA, USA). All other chemicals used were of analytical grade.

II. *Preparation of SN-38 NPs*

The SN-38 loaded NPs were fabricated by emulsification/solvent evaporation method[3,20]. In brief, 10 mg of PLGA 50:50 and 2 mg of SN-38 were added to DCM, which was stirred to ensure that all materials were dissolved. The dispersed phase was slowly dropped into aqueous solution of PVA (pH was adjusted to 3 by 0.1N HCl) using a high-speed homogenizer (ultra-turrax IKA, Wilmington, MA, USA) at 24,000 rpm. The formed o/w emulsion was gently stirred at room temperature by a magnetic stirrer for 3-5 hours to evaporate the organic solvent. The NPs were recovered by centrifuging (21,000 g, 30 min, Sigma 3k30, Germany), followed by washing thrice with deionised water. The produced suspension was freeze dried for 48 hours (Lyotrap plus, LYE Scientific Ltd, Oldham, UK) to obtain a fine powder of NPs. NPs without SN-38 were prepared by the same procedure. Freeze dried NPs were stored in a sealed glass vial and placed in desiccators kept at 4°C.

III. *Identification*

The identification was performed as a part of assay. The retention time of the major peak in the chromatogram of the test solution in three sequential replicate of injections was compared to that of standard solution. The same retention time of test and standard identified the same analyte.

IV. *Ion-pair Solution (Solution A)*

To prepare solution A, about 1.2 g of octane-1-sulfonic acid sodium salt was transferred to a suitable container and dissolved in 500 mL of water.

V. *Solution B*

To prepare solution B, 13.6 g of potassium dihydrogen phosphate (KH_2PO_4) was transferred to a suitable container and dissolved in 500 mL of water.

VI. *Mobile Phase*

Mobile phase consisted of a suitable filtered and degassed mixture of Ion-pair solution, solution B and acetonitrile (30:30:40, v/v/v). Then pH of mixture was adjusted to 3.0 with phosphoric acid.

VII. *Preparation of Stock and Standard Solutions*

An accurately weighed quantity of SN-38 standard (5 mg) was transferred into a 50-mL volumetric flask and dissolved in acetonitrile to obtain a stock standard solution with concentration of 100 µg/mL. Then other concentrations of standard solution for calibration curve were prepared by dilution of stock standard solution in a suitable volumetric flask with acetonitrile for L form and acetonitril-water (50:50, v/v) (pH of solution should be adjusted to10) for C form. Equal volumes (20 µL) of each standard solution and the test solution were injected into the chromatograph.

VIII. *Linearity and Range (Calibration Curve)*

The calibration curves were constructed by plotting the peak area ratio of the analytes over standard versus the concentrations spiked. Solutions of different concentrations (4-100 µg/mL) for construction of calibration plots were prepared from the stock solution as described above. The solutions of 4, 10, 30, 40, 50, 80 and 100 µg/mL of SN-38 were prepared by spiking of SN-38 L and C form at seven aforementioned concentrations.

IX. *Assay Preparation*

An accurately weighed portion of the freeze dried powder, presumably at a concentration similar to that

of the standard solution of SN-38, was transferred to a suitable volumetric flask, dissolved in and diluted with acetonitrile to volume, mixed and filtered to obtain a solution with concentration of the drug in the range of linearity previously described. The quantity of SN-38 in NPs (in mg), was calculated by the following formula:

$$100C\,(r_t/r_s)$$

in which C is the concentration of SN-38 reference standard in standard solution (in mg/mL); and r_t and r_s are the SN-38 peak responses (Peak area) of test and standard solution, respectively.

X. *Chromatographic System*

In this study, SN-38 was analyzed by a Knauer HPLC system (Germany) consisted of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2501 UV detector connected to a model Eurochrom 2000 integrator. The separation was performed using a C18 analytical column (Base deactivated packing H.P. 25 cm × 0.46 cm internal diameter, pore size 5 mm, Capital HPLC, UK) at 30°C using mobile phase (as mentioned above) at a flow rate of 1 mL/min. Each sample was injected into the column at a constant volume of 20 µL for an analysis run of 10 min (rapid). The UV detection wavelength was set at 265 nm for the chromatographic analyses of SN-38 NPs, respectively. System suitability solution chromatographed and recorded the peak responses (peak areas) as directed for procedure. The analytical column theoretical plate number and tailing factor of the analytes under different chromatographic conditions were calculated using USP methods[21].

XI. *Drug Encapsulation Efficiency*

The drug entrapped in the NPs was determined in triplicate by HPLC. A 10-mg sample of NPs powder was dissolved in 10 mL of acetonitrile. The solution was filtered using RC- 45/25 CHROMAFIL with pore size of 0.45 µm, Ø: 25 mm (MACHERY- NAGEL, Germany) into a vial. An aliquot of 20 µL was analyzed by the HPLC system to detect the SN38 concentration. The encapsulation efficiency of SN-38 was obtained as the mass ratio between the amount of SN-38 incorporated in NPs and that used in the NP preparation.

XII. *In vitro Release Study*

The release rate of SN-38 from the NPs was measured in phosphate buffered solution (pH 7.4) at 37°C by HPLC in triplicate. Briefly, 10 mg of NPs powder was poured in screw-capped tubes and dispersed in 10 mL of phosphate buffer solution (PBS). The pH of the monobasic potassium phosphate solution was adjusted by 0.2 N NaOH. Then the tubes were placed in an orbital shaker bath (WB14 Memmert, Schwabach, Germany), which was maintained at 37°C and shaken horizontally at 90 cycles/

min. At predetermined time intervals, the tubes were taken out of the water bath and centrifuged at 21,000 g for 30 min and the entire supernatant were collected for further SN-38 analysis by HPLC. Afterwards, the precipitated particles were re-suspended in 10 mL of fresh buffer (in order to maintain sink conditions) and put back into the shaker to continue the release measurement. The HPLC method used was the same as described above.

XIII. *Method Validation*

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications[21]. Typical analytical performance characteristics for the validation of procedures according to ICH Q2(R₁) guidelines described in this document include accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, robustness and system suitability test[22,23].

XIV. *Stability of Drug Solution*

Stability of the drug solution was determined based on the retention time and (%) recovery of SN-38 solution (4, 40 and 100 µg/mL). The drug solution was kept at room temperature (around 25°C) for three consecutive days.

RESULTS AND DISCUSSION

I. *Optimization of the Chromatographic Method*

An optimized reversed-phase HPLC method for the assessment of SN-38 in association with nanoparticulate carriers has been proposed. This study was exploited to provide a simple procedure with increasing chromatographic peak resolution, reduced running time and low cost of analysis. All these factors contribute to the establishment of an analytical method which permits the analysis of a large series of samples and prevents possible degradation due to long analysis time. The relatively symmetrical SN-38 peak has a retention time of 5.11 min. The chromatographic behavior of SN-38 was first studied using the method reported by Xuan *et al.*[13]. In this study, the SN-38 standard (40 µg/mL) was analyzed on a Nucleosil C18 (Phenomenex, 4.0 mm × 250 mm, 5 m) analytical column, NaH_2PO_4 (pH 3.1, 25 mM) and acetonitrile (50:50, v/v) mobile phase at a flow rate of 1 mL/min. It was found that retention times of C and L forms of SN-38 were very close and shifted as much as 5% among 6 replicate injections so that the base line separation was not completed when Nucleosil C18 was used. In order to prevent the retention time shift and increase the peak resolution of these two forms, changes in the

component of mobile phase were further investigated. In this study attempts to separate SN-38 L by varying the acetonitrile composition and in pH of the running buffer of the mobile phase in order from the C form interferences did not provide much improvement. It was found that increasing ion-pair agent and reducing buffer ratio led to minimal shift in retention time but significantly improved peak resolution. Hence, adding ion-pairing reagent octane-1-sulfonic acid sodium salt to the mobile phase changes the polarity gap between the two analytes, so as to improve chromatographic separation[24]. In the present study, we have chosen a mobile phase with 0.1 M potassium dihydrogen phosphate containing acetonitril and ion-pair agent at pH 3 which resulted in efficient separation between compounds with suitable retention time. The potassium dihydrogen phosphate buffer used in the mobile phase could control the pH value and ion strength, while the ion-paring agent octane sulphonic acid sodium salt would change the affinity of the C forms to column C18 particles, which would facilitate their separation.

As for other camptothecin class of compounds, SN-38 undergoes pH-dependent reversible hydrolysis of the active α-hydroxy-δ-lactone ring to form an inactive carboxylate derivative in aqueous solutions (PH ≥ 7) and plasma[13-19]. The L ring of SN-38 is unstable and converts to an open-ring C form under basic conditions. A survey of L-C structural conversion of SN-38 under the different pH conditions was performed using the defined ion-pair HPLC method to determine the desirable mobile phase pH with minimal structural conversion. As shown in Figure 3, 16.67% of SN-38 existed in C form and 83.3% in L form when pH of the diluents (water/acetonitrile = 50:50, v/v) was kept at 7. When pH of the diluent was changed to 10, about 100% of SN-38 was in C form (Figure 4). Once pH of the diluent was adjusted to 3, almost all the open C form of SN-38 converted to L structure. This pH-dependent structural conversion was found to be reversible. Consequently, pH of mobile phase was kept at 3 for the prevention of structural conversion during assay. Also addition of ion-pair solution in mobile phase increased the peak resolution and symmetric factor of two forms of SN-38. Representative chromatograms in Figure 3 show that C and L forms of SN-38 are base line separated with retention time of 2.9 ± 0.01 and 5.1 ± 0.01 min, respectively. As ultraviolet detection of the compound is more common than the fluorescent detection, in this study we developed a simple and reproducible method for determination of SN-38 in NPs containing SN-38 based on UV detection and isocratic mobile phase. This method was fast and efficient with simple sample preparation procedure and total analytes running time of less than 10 min, much shorter than those reported previously (about 20-30 min)[16,19]. Therefore, the risk of degradation and conversion of analytes can be significantly reduced during sample analysis. Note that the conversion between C and L does not occur in the mobile phase for at least 20 min (data not shown). Since
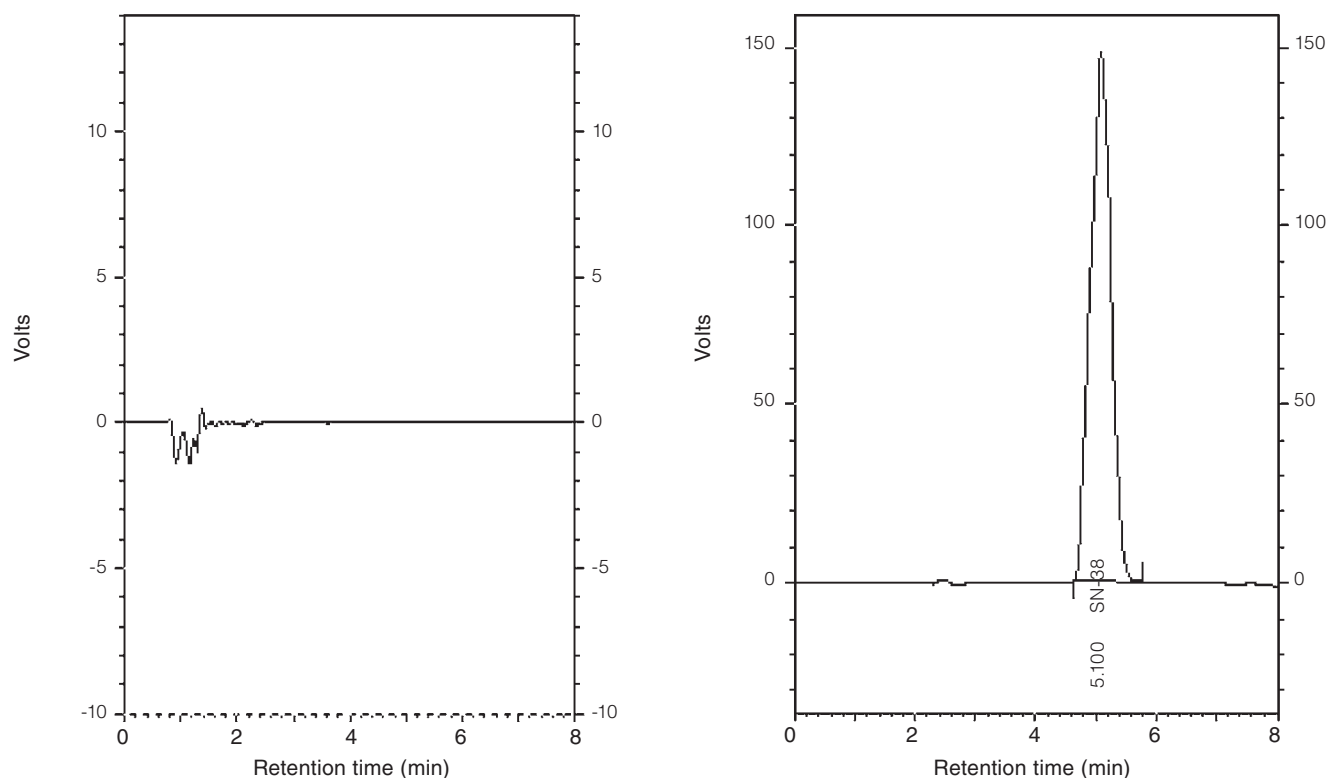
the retention times of these two forms are less than 20 min, it can be deduced that the real percentage of them has been calculated. A typical chromatogram for the
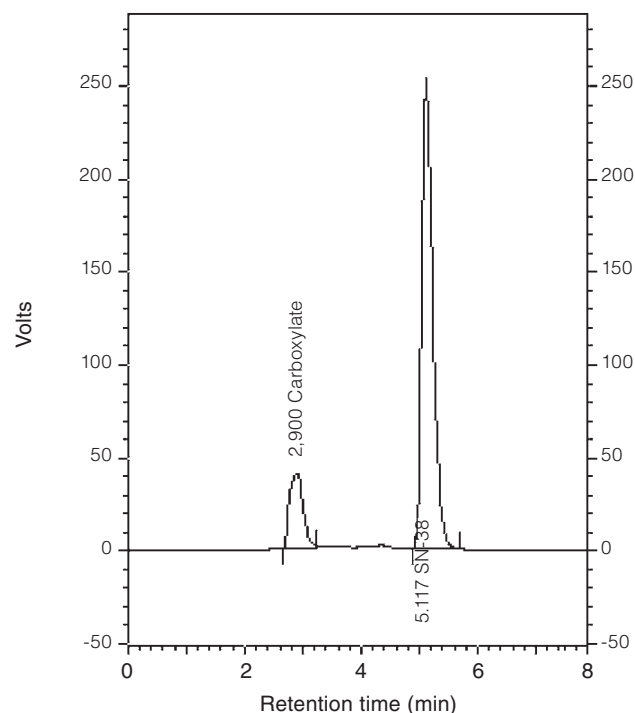
Figure 3. Representative HPLC chromatogram for SN-38 lactone and carboxylate form separation with resolution of 5.86 ± 0.12 (n = 3).
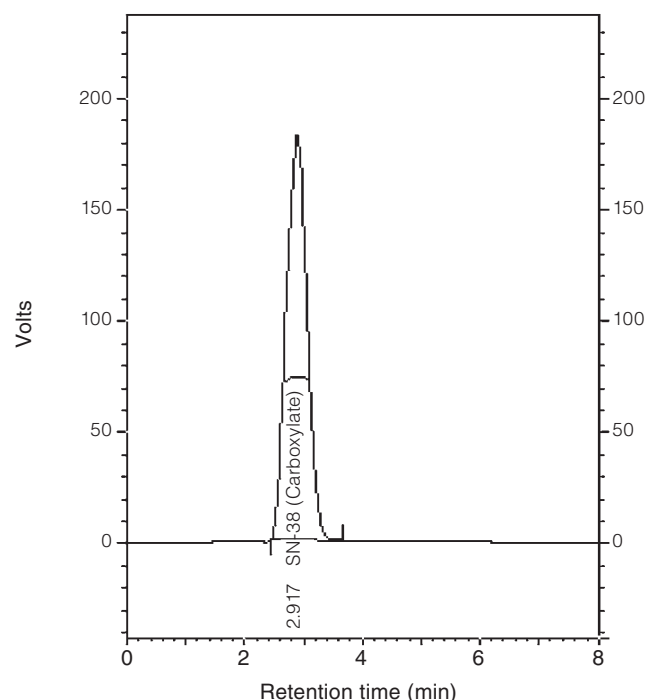
Figure 4. Representative HPLC chromatogram for SN-38 carboxylate form.

Figure 5. (a) HPLC chromatogram obtained from PLGA nanoparticles without SN-38. (b) HPLC chromatogram obtained from SN-38 loaded PLGA nanoparticles.

Table 1. Results of accuracy study for L form of SN-38

No.	Concentration (μg/mL)	Injection No.	Calculated × 100/Nominal (%)	Average recovery (%)	RSD (%)
		1	99.34		
1	4	2	101.10	100.30	0.88
		3	100.46		
		1	102.27		
2	40	2	101.94	102.11	0.16
		3	102.12		
		1	99.34		
3	100	2	98.86	99.57	0,85
		3	100.52		

proposed method is depicted in Figure 5b which shows lack of C forms of SN-38 in NPs.

II. *Method Validation*

(I) *Accuracy*

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure[16]. In this research, accuracy was calculated as the percentage of recovery by the assay of the known amount of SN-38 standard solution (L and C forms) which was added into synthetic mixture or placebo of PLGA NPs. The acceptance criteria of accuracy (% recovery) should be between 95.0 and 105.0%. The SN-38 assay showed recovery

percent of 99.57% to 102.11% with an overall average of 100.66% for L form (Table 1) and 99.86 to 100.59% with an overall average of 100.29% for C form (Table 2). The percent of relative standard deviation (% R.S.D.) for the triplicate measurements at each level was less than 1.0% which demonstrated a high level of accuracy.

(II) *Precision*

The precision was demonstrated at two levels: repeatability (intra-assay, intra-day or within day precision) and intermediate precision (inter-day or between-days precision). Each level of precision was investigated by 3 sequential replicate of injections of three concentrations of 4, 40 and 100 µg/mL of SN-38 NPs (pH was adjusted 10 for C form sample preparation). The intra- and inter-day variability or precision data were assessed by using standard solutions (L and C forms) prepared to produce solutions of three different concentrations of each drug. Repeatability

or intra-day precision was investigated by injecting nine replicate samples of each samples at each concentration. Inter-day precision were assessed by injecting the same three samples over three consecutive days. The precision was expressed as relative standard deviation (RSD) or coefficient of variation (CV). The results of repeatability and intermediate precision are shown in Table 3. The relative standard deviation (R.S.D.) values for absolute peak areas of SN-38 in samples were less than 1.2% for both intra-day precision and inter-day precision. These data demonstrated acceptable precision of the method.

(III) *Specificity*

Specificity was investigated by using different excipients without active substance and verifying the absence of interferences. The chromatogram of pure SN-38 was compared with the retention time and appearance of formulation excipients. In addition, empty NPs

Table 2. Results of accuracy study for C form of SN-38

No.	Concentration (µg/mL)	Injection No.	Calculated × 100/Nominal (%)	Average recovery (%)	RSD (%)
		1	101.23		
1	4	2	100.70	100.59	0.69
		3	99.86		
		1	100.17		
2	40	2	98.90	99.86	0.85
		3	100.52		
		1	100.53		
3	100	2	99.70	100.42	0.67
		3	101.05		

Table 3. The precision of the method in the range studied

Precision	Acceptance Criteria	Concentration (µg/mL)	RSD of peak area for L form	RSD of tR for L form	RSD of peak area for C form	RSD of tR for C form
Repeatability (intra-day precision)	The RSD of peak areas and retention time of SN-38 in the AVSS should not be more than 2%.	4	0.83	0.88	0.91	0.40
		40	0.71	0.56	0.70	1.1
		100	0.45	0.44	0.49	0.94
Intermediate precision (inter-day precision)		4	0.92	0.85	0.79	1.06
		40	0.64	0.78	0.63	0.82
		100	0.54	0.36	0.49	0.69

RSD: Relative Standard Deviation; AVSS: Analytical Validation Standard Solution
Acceptance Criteria: The RSD of peak areas and retention time of SN-38 in the *AVSS* should not be more than 2%.

were prepared in the same way for SN-38 NPs. The main purpose was to detect any retention peak due to single or combination of isolate polymer. No peak was observed in the chromatogram of each excipient and empty NPs at the retention time of SN-38 NPs (L form) (Figure 5a and 5b) and C form (Figure 4). Our result indicates specificity of the method.

(IV) *Limits of Detection and Quantitation*

The LOD was determined by analyzing SN-38 standards in six successive injections at different concentration levels in decreasing order until signal to noise (S/N) ratio reached about 3. The LOQ was established in the same way as LOD, except that the S/N ratio was about 10. The LOD of this method for L and C forms were 0.08 μg/mL (% R.S.D. = 6.58) and 0.02 μg/mL (% R.S.D. = 3.61), respectively. The LOQ of this method for L and C forms were 0.26 μg/mL (% R.S.D. = 2.24), and 0.12 μg/mL (% R.S.D. = 1.92), respectively.

(V) *Linearity and Range (Calibration Curve)*

Linearity was established by least squares linear regression analysis of the calibration curve[25]. As in this study the concentration of test solution was 40 μg/mL, so the Analytical Validation Standard Solution (AVSS) covered from 10 to 250% of assay concentration (4 to 100 μg/mL, n = 7) seems suitable for determination of SN-38 loaded PLGA NPs. Peak areas of SN-38 were plotted versus their respective concentrations and linear regression analysis performed on the resultant curves. The mobile phase was filtered through a 0.45-μm membrane

filter and delivered at 1 mL/min for column equilibration; the baseline was monitored continuously during this process. It was found that in the concentration range, the detector response of SN-38 was linear with a correlation coefficient greater than 0.999 for L form and 0.9998 for C form.

(VI) *Robustness*

The robustness of the method was investigated under a variety of conditions, including changes of pH of the eluent, flow rate, column temperature, and ratio (acetonitrile to buffer solution) of the mobile phase[25]. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and analytical operators has proven that the method is robust (Table 4).

Overall, the method demonstrated sufficient robustness and suitability for the analysis of SN-38 NPs under the deliberately altered HPLC conditions.

(VII) *System Suitability Test*

System suitability tests are an integral part of liquid chromatographic methods. They are used to verify that resolution and reproducibility of the chromatographic system are adequate for the analysis[21]. On the basis our analytical validation results, the values for column efficiency (N), tailing factor (T), resolution (R), and RSD (%) were calculated. The column efficiency was more than 1000 theoretical plates for SN-38 L and C forms. The tailing factor, T, a measure of peak symmetry was determined as 1.01 for both forms which is less than usual

Table 4. Robustness of the method by changing the experimental parameters on the performance of chromatographic system

Variable Parameters	Modification	Recovery (%) for L form	RSD (%) for L form	Recovery (%) for C form	RSD (%) for C form
Buffer composition* (v/v)	30:34:36	100.02		98.8	
	30:30:40	100.3	0.30	100.1	0.68
	30:26:44	99.7		99.1	
pH of mobile phase	2.7	99.25		101.04	
	3	100.6	0.90	100.3	1.07
	3.3	98.9		98.9	
Flow rate (mL/min)	0.9	99.8		97.9	
	1	98.9	0.66	99.1	0.84
	1.1	100.2		99.5	
Temperature (°C)	27	101.02		100.7	
	30	99.6	0.70	101.1 99.7	0.71
	33	100.4			

The concentration of the solution analyzed was 40 μg/mL.
*: Ion-pair solution, solution B and acetonitrile

pharmacopoeial standard (less than 2.0). The relative standard deviation for replicate injections was also less than 2.0%. The resolution, R, was specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system. As shown in Figure 3, the resolution for L and C forms of SN-38 is 5.86 ± 0.12 (n = 3).

(VIII) *Stability of Drug Solution*

The three-day percentage relative standard deviation of the retention time and (%) recovery of SN-38 C and L forms was less than 1% (Table 5). No significant degradation was observed within three days, indicating that SN-38 C and L forms solutions were stable for at least three days at room temperature.

III. *Application of the Method*

The proposed method was applied to study SN-38 association with PLGA NPs produced by modified emulsification solvent evaporation method. Previous experiments were done to establish the ideal formulation and to characterize those NPs. The encapsulation efficiency (EE) of SN-38 NPs was more than 80% due to high lipophylicity of SN-38. No SN-38 C peak was detected in SN-38 loaded PLGA NPs. The EE was also evaluated during the shelf life in order to assess the SN-38 stability associated with the carriers. The EE did not change after 6 month of storage at 4°C. It was demonstrated that no other NP components interfered with SN-38 determination. Figure 6 shows the profile of SN-38 release from

the NPs in PBS (pH 7.4) as a suitable application of this method. SN-38 release was characterized by an immediate burst effect. Up to 10% of SN-38 was released from NPs within 1day of contact with the release media and a cumulative release of 35% occurred after 10 days. This was expected because of its highly hydrophobic nature which prevented the drug diffusion from the polymer matrix into the aqueous solution. We also applied this validated HPLC method to determine pharmacokinetic of SN-38-loaded PLGA NPs in rat plasma (data not shown). HPLC analysis in the *in vitro* release test confirmed that the release drug had the same retention time as the free SN38, indicating that the chemical structure of SN38 was not damaged during NP preparation. Nevertheless, to further verify whether the released SN38 was still pharmacologically active and to evaluate the potential cytotoxicity of SN38-free drug and SN-38 NPs, an *in vitro*
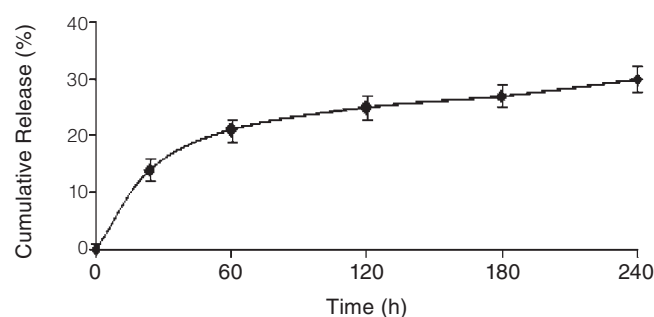
Figure 6. *In vitro* release curve of SN-38 loaded PLGA nanoparticles using the proposed HPLC method.

Table 5. Stability study of SN-38 solution

| Day | Concentration (μg/mL) | L form | | | C form | | |
		RSD of t_R (%)	Recovery (%)	RSD (%)	RSD of t_R (%)	Recovery (%)	RSD (%)
1	4		99.7, 101.0, 100.1	0.66		100.01, 98.2, 98.7	0.93
	40	0.11	100.2, 99.1, 98.5	0.86	0.52	98.5, 99.8, 99	0.65
	100		100.1, 98.7, 100.5	0.95		101, 99.6, 99.5	0.83
2	4		100.2, 99.8, 100.6	0.40		99.2, 98.7, 98.1	0.55
	40	0.29	99.9, 100.1, 101.7	0.98	0.59	98.8, 100.1, 99	0.70
	100		100.1, 98.7, 98.9	0.76		100, 99.1, 98.8	0.62
3	4		100.2, 99.4, 98.9	0.65		97.9, 98.6, 99	0.56
	40	0.48	98.6, 99.2, 98.1	0.56	0.64	98.7, 99.1, 99.3	0.30
	100		98.2, 99.1, 98.7	0.46		99.2, 8.05,100.01	0.98

n = 3

cytotoxicity test against HT29 cells (as a colon carcinoma cell lines) was performed. The obtained results confirmed that SN-38 loaded PLGA NPs were more cytotoxic than SN38-free drug (data not shown).

CONCLUSIONS

This study has described a new, rapid and validated ion-pair RP-HPLC method for simultaneous determination of L and C forms of SN-38 according to the ICH guidelines. All parameters were within the limits proposed by the guidelines for pharmaceutical formulations, indicating that this method is specific, precise, accurate and robust with low detection and quantitation limits. Furthermore, suitable application for SN-38 *in vitro* analysis can be assumed during formulation development and characterization. The proposed method was used to predict the encapsulation efficiency, release profile, cytotoxicity and biodistribution study of SN-38 from nanoparticulate carriers.

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