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Research Article

THE DEVELOPMENT AND VALIDATION OF A CHIRAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF (R)-ENANTIOMER IN 7-ETHYL-10-HYDROXYCAMPTOTHECIN (SN-38)

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ABSTRACT

Objective: The objective of the method was to develop a new and simple, rapid, isocratic, normal phase chiral HPLC method for the enantiomeric separation of (S)-7-ethyl-10-hydroxycamptothecin(SN-38){(S)-4,11-Diethyl-4,9-dihydroxy-1H-pyrano[3',4;6,7] indolizino[1,2-b]quinoline-3,14(4H,12H)-dione, an anti-cancer drug substance.

Methods: In this method, a Chiralpak IC (Daicel Chemical Industries, Ltd., Tokyo, Japan) (immobilized polysaccharide chiral stationary phase) column with a mobile phase consisting of *n*-hexane:ethanol (50:50 v/v) at a flow rate of 1.0 mL/min was used. The elution was monitored at 225 nm and column oven temperature at 40 °C.

Results: The limit of detection and limit of quantification of R-enantiomer were found to be 0.032 µg/mL and 0.07 µg/mL respectively for 10 µL injection volume. The sample solution and mobile phase were found to be stable for at least 48 hr. The linearity was showed a regression coefficient of 0.9993. The resolution between both the enantiomers was greater than 3.5 in the optimized method. The developed method was extensively validated and proved to be robust, enantioselective, accurate, precise, and suitable for quantitative determination of (R)-enantiomer in bulk drug substance and product.

Conclusion: The developed method was simple, fast, accurate and precise and hence could be applied for routine quality control analysis for quantitative determination of (R)-enantiomer in bulk drug substance and product.

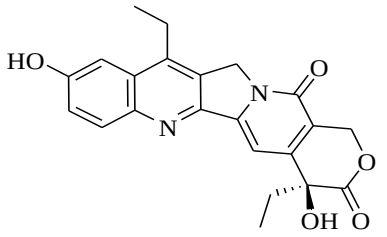
Keywords: 7-Ethyl-10-hydroxycamptothecin; (R)-enantiomer; validation; Anti-cancer activity; quantification; HPLC.

INTRODUCTION

7-Ethyl-10-hydroxycamptothecin(SN-38)(4**S**)-4,11-Diethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinoline-3,14(4H,12H)dione (Fig. 1) is the active metabolite of Irinotecan (CPT-11), a topoisomerase I inhibitor commercially available as Camptosar. SN-38 is a very capable anticancer drug used for the treatment of metastatic colon rectal cancer. SN-38 is a total synthetic anticancer drug. SN-38, shows much stronger cytotoxicity *in vitro* than CPT-11. SN-38 is approximately 200-2000 fold more cytotoxic than CPT-11. The formulation contains liposomes of uniform size distribution (<200 nm), and it is easytouse. Drug entrapment efficiency of the formulation is > 95%. Long term stability studies specify that the lyophilized LE-SN-38 is chemically and physically stable for at least 6 months at 2 - 8 °C. In preclinical studies, LE-SN-38 has shown capable results in terms of increased cytotoxicity against various tumor cell lines and better therapeutic efficacy towards xenograft mouse models compared to CPT-11. However, CPT-11 feebly inhibited DNA synthesis independently of time with coincident inhibition of the total thymidine uptake by the cells. By alkaline and neutral elution assays, it was demonstrated that SN-38 caused much more frequent DNA single strand breaks in P388 cells than did CPT-11. The same content of SN-38 and a similar frequency of single strand breaks were detected in the cells treated with SN-38 at 0.1 µm or with CPT-11 at 100 µm. Therefore, single strand breaks by CPT-11 appear to be due to SN-38 produced from CPT-11 in cells. These results designate that CPT-11 itself possesses a marginal antiproliferative effect but that SN-38 plays an vital role in the mechanism of action of CPT-11 [1- 6].

SN-38, a novel compound with an indole alkaloid structure, was clinically tested as a therapeutic agent by oral administration in the treatment of metastatic colon rectal cancer. The (S)-enantiomer has turned out to be a potent breast cancer resistance protein (BCRP) ABCG2 in the human lung cancer cells [5].No HPLC method is available in the literature for the chiral analysis of SN-38.

SN-38 is the first synthetic commercial product that has been developed and marketed.



7-Ethyl-10-hydroxycamptothecin

Fig. 1: Chemical structure of 7-ethyl-10-hydroxycamptothecin

SN-38 is produced as a single isomer by a total synthesis of 13 steps and (R)-enantiomer could be present as a chiral impurity that is obtained in small amounts during the synthesis of SN-38. Companies developing chiral drugs, where only one enantiomer is responsible for bioactivity, have to make sure that the process for their production is planned and optimized to minimize the formation or occurrence of the unwanted enantiomer to trace or below detection levels before taking the drug for toxicological, metabolic physical and pharmacokinetic, evaluation and establish its therapeutic benefits [7-8].As per the International Conference on Harmonization (ICH) guidelines, all unknown impurities which are forming at a level more than 0.1% with respect to drug substance or API, they should be identified, synthesized and characterized

Thoroughly [9-11].Such stringent levels of purity require sensitive and consistent analytic methods that allow for the detection of these unwanted isomers [12]. To the best of our knowledge, no chiral

HPLC method is available in the literature for the chiral analysis of SN-38 [13-17].

Separation of enantiomers has become very significant in analytical chemistry, particularly in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite dissimilar pharmacokinetics and different pharmacological or toxicological effects [18].

In current years, research has been intensified to understand the aspects of the molecular mechanism for stereoselective biological activities of the chiral molecules. The development of analytical methods for the quantitative analysis of chiral materials and for the assessment of enantiomeric purity is enormously challenging due to the fact that enantiomers possess virtually identical properties [8].Recently, much work has been reported describing the use of chiral stationary phases, in conjugation with HPLC, as a way to separate and thereby individually quantitative the enantiomers of an enantiomeric pair [19-20]. The chiral nature of the drug has made the importance to develop the chiral HPLC method for the enantiomeric purity and quantitative determination of undesired isomer.

An easy and fast isocratic LC method is often more preferred in ordinary lab. Polysaccharide chiral stationary phases are pretty popular with wide recognition for direct resolution of enantiomers. A literature survey revealed that there is no HPLC method for separation of the (S) and (R) enantiomers along with the quantitative estimation of (R)-SN-38. We have initiated some work to develop a normal phase HPLC method for the quantitative determination of (R)-enantiomer in SN-38. The present research work deals with rapid, simple, precise and robust enantioselective isocratic chiral LC method for the enantiomeric separation of SN-38 using an immobilized polysaccharide based chiral stationary phase (Chiralpak IC). This paper deals with the validation of determination of the (R)-enantiomer in SN-38 drug substance and drug product.

MATERIALS AND METHODS

SN-38 was synthesized by a total synthesis. (R)-Enantiomer was prepared by using preparative HPLC in the laboratory. HPLC grade *n*-hexane was procured from Rankem (New Delhi, India). The HPLC grade ethanol was procured from Commercial Alcohol (Mumbai, India). Stock solution was prepared in ethanol at a concentration of 0.35 mg/mL.

Methods

Instrumentation

High Performance Liquid Chromatography (HPLC)

A Shimadzu HPLC system LC-2010 CHT with a photo diode array detector (PDA) was used for method development and validation. The output signal was observed and processed using LC solution software.

Preparation of Standard and Sample Solutions

The stock solutions of SN-38 (0.35 mg/mL) and (R)-enantiomer (0.35 mg/mL) were prepared by dissolving an appropriate amount of ingredients in diluent (ethanol). For quantification of (R)-enantiomer in SN-38, a solution of 0.35 mg/mL concentration was used.

Chromatographic Conditions

Analysis was carried out by using a chiral stationary phase, Chiralpak IC, 250 mm × 4.6 mm, 5 µm (Daicel Chemical Industries, Ltd., Tokyo, Japan) column. The Isocratic mobile phase consists of *n*-hexane and ethanol (50:50 v/v). The flow rate of the mobile phase was 1.0 mg/mL. A wavelength of 225 nm was found to be suitable for this analysis. The column temperature was maintained at 40°C and the injection volume was 10 µL.

Method Development

The method development strategies adopted using chiral Pack IC column involves various experiments based on nature and structure of compound. The design of mobile phase comprises a combination of alkane and polar alcohols based on normal or polar interactive modes. Initiated the screening analysis with the above combination of trials to derive best suitable column and mobile phase conditions.

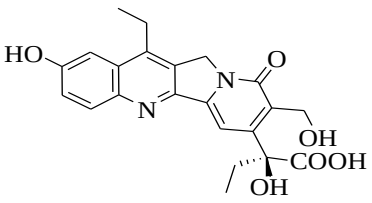
The racemic mixture was prepared by physical mixing of equal proportions of (R) and (S) SN-38 (0.5 mg of each sample). A 0.35 mg/mL solution of racemic mixture was prepared in ethanol and used for the method development. To develop the suitable chiral HPLC method for the separation of the enantiomers of SN-38, various mobile phases were employed.

Numerous experiments were carried out in the normal phase using various chiral columns and mobile phases to develop the suitable chiral HPLC method for the separation of the enantiomers of SN-38. While using the chromatographic conditions like chiralcel OD(H) and chiralcel OD columns with flow rate 1.0 mL/min and mobile phase of n-hexane:IPA (50:50) (v/v) mixture, it has been noticed that the enantiomers of SN-38 were eluted as broad peaks and the resolution (Rs) between isomers is very low. Insufficient separation was observed on these chiral stationary phases (CSP). However an improvement in peak shapes was observed when Chiralpak IC column with mobile phase of n-hexane, IPA (50:50) (v/v) mixture and same flow rate are used, but still, there are certain constraints like improper separation (Rs < 1.2) between the enantiomers were observed. The next attempt was made on this amylose based CSP, wherein ethanol was used as a polar organic modifier in place of IPA, using the mobile phase consisting of n-hexane:ethanol (60:40), which was provided considerable separation between the isomers with longer retention times. Further trial was continued on the same CSP by increasing the polar ethanol percentage from 40% to 50% and addition of column oven temperature at 40 °C. With these two changes, this gave reasonably good peak shape with lesser retention time as well as good resolution between the analyte peaks (Rs > 3).

In the present optimized method, the typical retention times of (R) and (S) SN-38 are eluted at 8.17 min and 10.19 min respectively (Fig. 4). The peak purity of SN-38 is found to be homogeneous in all spiked samples. The resolution (Rs) between the two enantiomers was about 4.20. Diluent ethanol was used as blank and there was no interference of the blank with (R) and (S) isomers of SN-38. The developed method is found to be selective from process related impurities.

Finally, the resolution was found to be more than 3 for the separation of (R) and (S) isomers of SN-38 with the mobile phase consisting of n-Hexane, ethanol in the ratio of 50:50 (v/v) and column oven temperature at 40 °C. The elution was monitored at wavelength 225 nm. Then, the same conditions were maintained for the determination of (R)-SN-38 in (S)-SN-38.

When SN-38 compound was subjected to base (1 N NaOH for 24 hours at temperature 60 °C), the lactone ring of SN-38 is open in an alkaline environment and the acid form of SN-38 (ring-opened form, Fig. 2) is analyzed using the same chiral normal phase method. The hydrolyzed product is clearly separated from the two enantiomers (Fig. 3). Hence there is no interference with the quantification of R and S enantiomers of SN-38.



Acid form of (S)-7-ethyl-10-hydroxycamptothecin

Fig. 2: Hydrolyzed product (ring-opened form) of SN-38

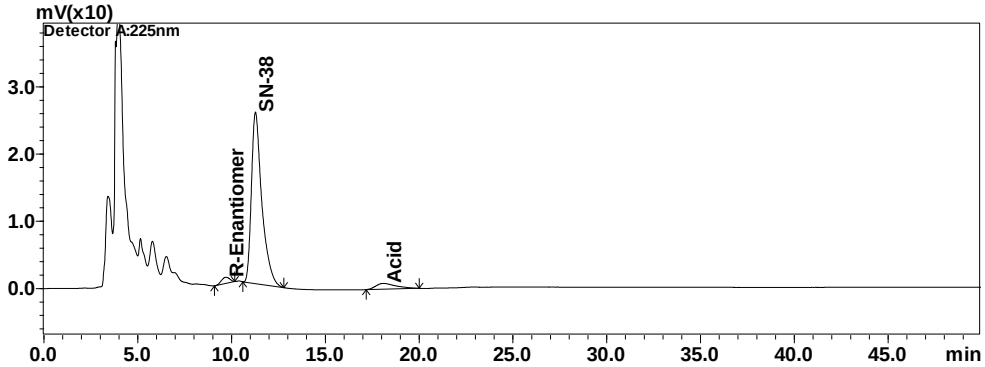


Fig. 3: Typical HPLC chromatogram of SN-38 compound when subjected to base (1 N NaOH for 24 hours at temperature 60 °C)

Analytical Method Validation

System Suitability

The system suitability test was determined by injecting racemic mixture containing equal quantity of (R) and(S) enantiomers. Since the enantiomers form a critical pair of peaks in the chromatogram, the qualification criteria was resolution between two enantiomers, shown to be not less than 3.5 and tailing factor should not exceed 1.5.

Precision

Method reproducibility was determined by measuring repeatability and intermediate precision (between day precision) of retention times and peak areas for each enantiomer.

In order to determine the repeatability of the method, replicate injections (n = 6) of a 0.35 mg/mL solution containing SN-38 spiked with (R)-enantiomer (0.1%) was carried out. The intermediate precision was also evaluated over two days by performing six successive injections each day.

Linearity of (R)-enantiomer

Linearity was assessed by preparing six calibration sample solutions of (R)-enantiomer covering from 0.07 µg/mL (LOQ) to 0.525 µg/mL (LOQ (20%) to 150%) of the permitted maximum level of the (R)-enantiomer (0.07, 0.0875, 0.175, 0.265, 0.350, 0.435 and 0.525 µg/mL i.e. 20% (LOQ), 25%, 50%, 75%, 100%, 125% and 150%) and prepared in ethanol from (R)-enantiomer stock solution.

Regression curve was achieved by plotting peak area versus concentration, using the least squares method. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated. The upper and lower levels of the range were also established.

Quantification of (R)-enantiomer in Bulk Drug Substance & Product

The bulk drug substance and product did not show the presence of (R)-enantiomer; therefore standard addition and recovery experiment was performed to determine the accuracy of the present method for the quantification of (R)-enantiomer.

The study was performed in triplicate at 0.05%, 0.1% and 0.15% of the SN-38 target analyte concentration. The recovery of (R)-enantiomer was calculated by determining recovery of the spiked amount of (R)-enantiomer in SN-38.

Sensitivity (Limit of Detection & Limit of Quantification of (R)-enantiomer)

Limit of detection and limit of quantification of (R)-enantiomer were achieved by injecting a series of dilute solutions of (R)-enantiomer [21].

The sensitivity of the method was determined by establishing the limit of detection (LOD) and limit of quantification (LOQ) for (R)-enantiomer, which was estimated using slope method(ICH Q2 (R1)) [22]by injecting a sequence of dilute solutions of a known concentration.

The precision study was performed at the LOQ level by analyzing six test solutions prepared at LOQ level and calculating the percentage relative standard deviation of area.

Robustness of the Method

To determine robustness of the method, experimental conditions were intentionally altered, and chromatographic resolution between enantiomers was evaluated.

The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution, 0.1 units were changed from0.9 to 1.1 mL/min. The effect of column temperature on resolution of both isomers was studied at38 °C and 42 °C instead of40 °C while keeping mobile phase constant. The effect of change in percent of ethanol on resolution was studied by varying from −5 to +5% while the other parameters keeping constant. In the varied chromatographic conditions viz. flow rate, column temperature and mobile phase composition, the resolution between the peaks of isomers was found to be more than 3 illustrating the robustness of the method.

Solution Stability and Mobile Phase Stability

Stability of SN-38in solution at analyte concentration was carried out by leaving the solution in tightly capped volumetric flask at room temperature on a laboratory worktable for 48 h. The content of (R)-enantiomer was checked at 6 h intervals up to the study period.

The mobile phase stability study was also carried out for 48 h by assessing the content of (R)-enantiomer in SN-38. The same mobile phase was used for the 48 h during the entire study period.

RESULTS AND DISCUSSION

Development and Optimization of HPLC Conditions

The mechanism of separation in direct chiral separation methods is the interaction of chiral stationary phase (CSP) with enantiomer that is analyte to form short-lived, transient diastereomeric complexes. The complexes are formed as a result of hydrogen bonding, dipole-dipole interactions, pi bonding, electrostatic interactions, and inclusion complexation [23].

The CSP that gave the best separation was Chiralpak IC which is cellulose tris(3,5-dichlorophenylcarbamate) with immobilized polysaccharide based CSP on silica gel. The separation of enantiomers on Chiralpak IC was due to the interaction between the solute and the polar carbamate group on the CSP. The carbamate group on the CSP interacts with the solute through hydrogen bonding using C=O and NH groups present in the CSP and C=O and OH in the SN-38. In addition the dipole-dipole interaction occurs between the C=O group on the CSP and C=O group on the SN-38.

The immobilized polysaccharide based stationary phase in Chiralpak IC column has higher selectivity than protein based (chiral AGP) and amylose based (Chiralpak AD-H) columns, being suitable for the enantioselective separation and accurate quantification of (R)-SN-38. Another advantage of Chiralpak IC column is their greater stability under normal operation than other Daicel chiral columns. Immobilized column have excellent stability to strong solvents like tetrahydrofuran, ethyl acetate, and chlorinated solvents. Using immobilized stationary phase columns allows a great freedom of solvent choices.

A representative chromatogram of the enantiomeric resolution of SN-38 was shown in Fig. 4. An excellent resolution (Rs = 4.20) between two enantiomers and ideal peak shape with tailing factor 1.13 was obtained. The system suitability test results of the chiral liquid chromatographic method on Chiralpak IC are presented in (Table 1).

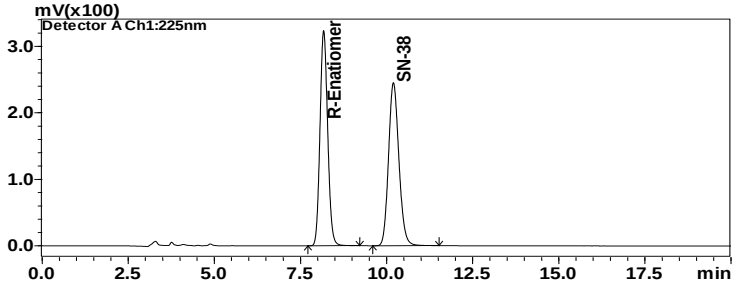


Fig. 4: Typical HPLC chromatogram of (R)-enantiomer and SN-38 (1:1)

Table 1: Data of system suitability and specificity

Parameter	(R)-Enantiomer	SN-38
Retention time (min)	8.17	10.19
Relative retention time	0.80	1.00
Resolution (Rs)	-	4.20
USP tailing factor (T)	1.10	1.13
No. of theoretical plates	6337	5439
%RSD retention time	0.06	-
%RSD peak area	1.09	-

USP: United States Pharmacopeia

Validation Results

In the precision study, the percentage relative standard deviation (RSD) was less than 0.5% for the retention times of the enantiomers, 0.95% for SN-38 peak area and 0.86% for peak area of (R)-enantiomer. In the intermediate precision study, the results showed that RSD values were in the same order of magnitude than those obtained for repeatability and the results are presented in Table 2.

Table 2: Data of Precision studies

Compound Name (µg/mL)	Spiked Concentration	Measured concentration	RSD (%)
Repeatability (n=6)	precision (n=6)	Intermediate	
R-Enantiomer	0.35	0.43	1.84

The limit of detection (LOD) and limit of quantification (LOQ) concentration were estimated to be 0.035 and 0.07 µg/mL for (R)-enantiomer, when signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for (R)-enantiomer at limit of quantification was less than 2.0 percentage relative standard deviation and the results are given in Table 3.

Table 3: Data of LOD & LOQ

Compound name	LOD	S/N ratio	LOQ	S/N ratio
Impurity A	0.035 µg/mL	11:1	0.07 µg/mL	17:1

The described method was linear in the range of 0.07 to 0.525 µg/mL for (R)-enantiomer in SN-38. The calibration curve was drawn by plotting the peak area of (R)-enantiomer vs its corresponding concentration with correlation coefficient of 0.999(Table 4). The equation of the calibration curve for (R)-enantiomer wasy =73701063.2935 x –73.3017 (Fig. 5).

Table 4: Linearity data of (R)-enantiomer

S. No.	Concentration (% of spec level)	Concentration (mg/mL)	Average area
1	LOQ	0.000070	5122
2	25.0	0.0000875	6536
3	50.0	0.000175	13039
4	75.0%	0.000263	18816
5	100.0%	0.000350	25275
6	125.0%	0.000438	32590
7	150.0%	0.000525	38694
	Slope-73701063.2935		
	Intercept–73.302		
	Correlation0.9997		
	Coefficient of determination (R^2)0.9993		

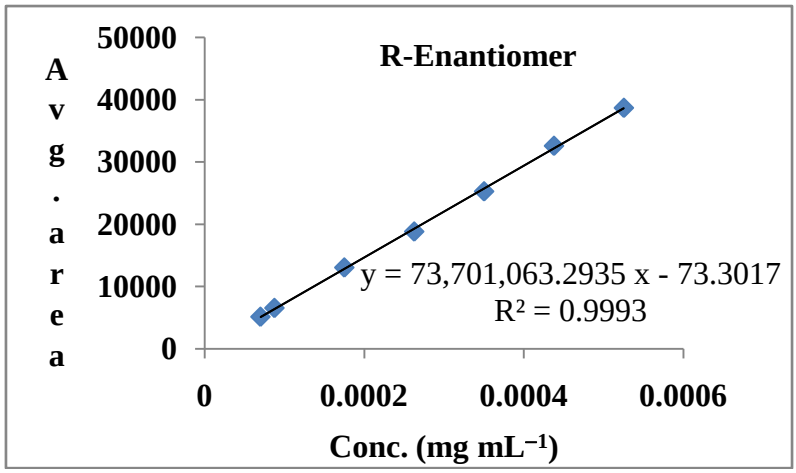


Fig. 5: Linearity graph of (R)-enantiomer of SN-38

The addition and recovery experiments were carried out for (R) enantiomer in bulk samples in triplicate at 0.05, 0.10 and 0.15% of the analyte concentration. Percentage recovery ranged from 99.3 to 102.9%. The recovery results are depicted in Table 5

Table 5: Recovery studies of (R)-enantiomer

Actual concentration (µg mL^{-1})	Calculated Concentration (n=3)	Standard Deviation	RSD (%)	Recovery
0.175	0.180	0.25	0.54	102.9
0.350	0.348	0.18	0.08	99.33
0.525	0.528	0.72	0.14	100.65

A HPLC chromatogram of (R)-enantiomer is shown in Fig. 6, a HPLC chromatogram of spiked (R)-enantiomer at 0.1% level in SN-38 sample is shown in Fig. 7 and a HPLC chromatogram of SN-38 is shown in Fig. 8.

The chromatographic resolution of the SN-38 and (R)-enantiomer peaks was used to assess the method robustness under modified conditions. The resolution between SN-38 and (R)-enantiomer was >3 under all separation conditions tested (Table 6), demonstrating sufficient robustness.

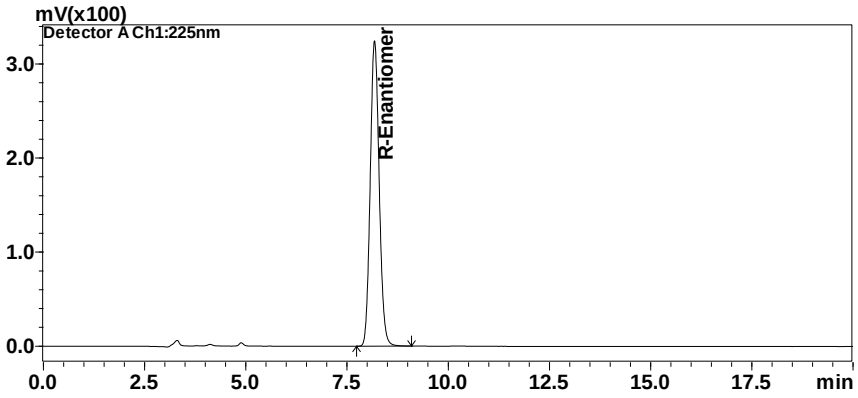


Fig. 6: Typical HPLC chromatogram of (R)-enantiomer of SN-38

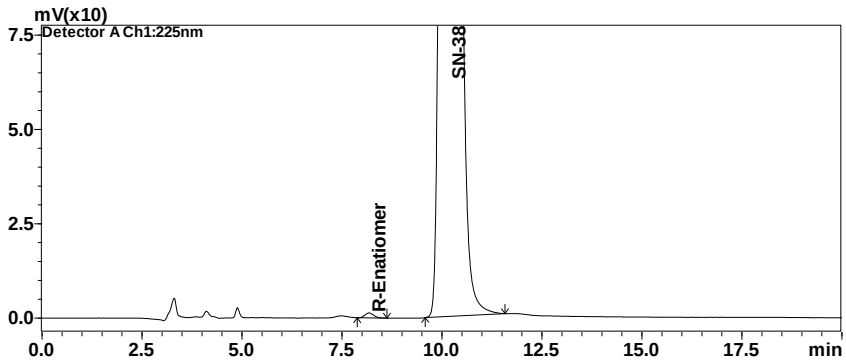


Fig. 7: Typical HPLC chromatogram of SN-38 spiked with (R)-enantiomer at 0.1% specification level.

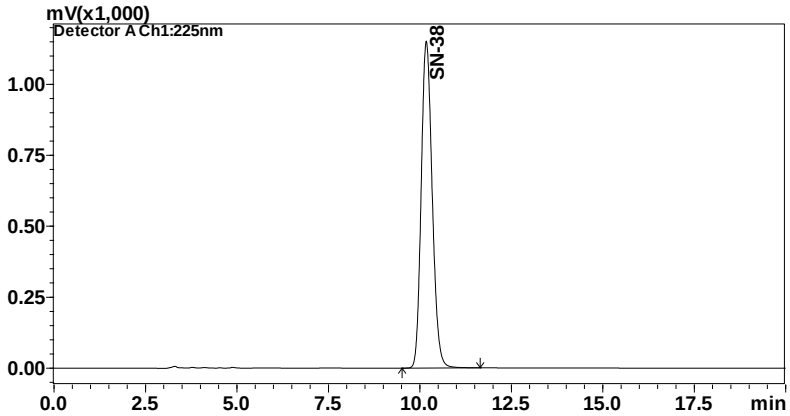


Fig. 8: Typical HPLC chromatogram of SN-38 Sample

Table 6: Data of robustness of the chiral LC method

Parameter	Variation altered	Relative retention time of R-enantiomer	Resolution
Flow rate	0.9 mL	0.80	4.44
	1.0 mL	0.80	4.27
	1.1 mL	0.80	4.09
Column oven	38°C	0.80	4.19
Temperature	40°C	0.80	4.27
	42°C	0.80	4.32
Mobile phase	55: 45	0.78	4.64
Composition	50: 50	0.80	4.27
(Hexane: Ethanol)	45:55	0.81	3.90

The percentage relative standard deviation of (R)-enantiomer content during solution stability and mobile phase stability experiments wasbelow0.5%. Hence SN-38 sample solution and mobile phase were stable for at least 48 h.

CONCLUSION

A simple, rapid and precise normal phase chiral HPLC method has been developed and validated for the enantiomeric separation of SN-38. Chiralpak IC was found to be selective for the enantiomers of the drug. The completely validated method was showing satisfactory data for all the method validation parameters tested. The developed method can be conveniently used by the quality control department for the quantitative determination of chiral impurity (R-enantiomer) in the bulk material. The developed method is more rapid and enantioselective. The method demonstrates right order of elution of (R)-enantiomer and (S)-enantiomer. The developed method is more suitable with respective to resolution (> 3), number of theoretical plates (>5000), United States Pharmacopeia (USP) tailing (<1.5) and percentage recovery of the (R)-enantiomer between 99.3 and 102.9%.

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