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Article

The Development and Validation of a Chiral High Performance Liquid Chromatography Method for the Identification and Quantification of (*R*)-Enantiomer in 10-Hydroxycamptothecin

***A. Venkateshwarlu,*[*,a,b] *A. V. Rama Rao,*[a] *Mukkanti Khagga*[b] *and S. V. Subba Reddy*[a]**

[a]*Analytical Development Laboratory, Avra Laboratories Private Limited, Plot No. A-21, Road No. 10, IDA Nacharam, 500076 Hyderabad, India*

[b]*Institute of Science & Technology, Jawaharlal Nehru Technological University, 500085 Hyderabad, India*

Um novo método isocrático, rápido e simples da cromatografia líquida de alta performance (HPLC) de fase normal quiral foi desenvolvido e validado para a separação enantiomérica do ácido (*S*)-10-hidroxicamptotecin (10 HCTN), ((4S)-4-etil-4,9-dihidro-1H-pirano [3',4':6,7] indolizina [1,2-b] quinolina-3, 14 (4H, 12H)-diona), um fármaco anti-cancerígeno. Os enantiômeros de 10 HCTN foram separados em uma coluna Chiralpak IC (fase estacionária quiral derivada de polissacarídeo imobilizado), utilizando uma fase móvel que consiste de *n*-hexano:etanol (50:50 v/v) em uma vazão de 1,0 mL min^{-1}. A resolução entre os dois enantiômeros foi superior a 3 usando o método otimizado. O método desenvolvido foi validado e mostrou serrobusto, exato, enantiosseletivo, preciso e adequado para a determinação quantitativa do enantiômero-(*R*) tanto na matéria prima quanto no fármaco.

A new and simple, rapid, isocratic, normal phase chiral high performance liquid chromatography (HPLC) method was developed and validated for the enantiomeric separation of (*S*)-10-hydroxycamptothecin (10-HCTN), ((4S)-4-ethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7] indolizino[1,2-b] quinoline-3,14(4H,12H)-dione), an anti-cancer drug substance. The enantiomers of 10-HCTN were resolved on a Chiralpak IC (immobilized polysaccharide chiral stationary phase) column using a mobile phase consisting of *n*-hexane:ethanol (50:50 v/v) at a flow rate of 1.0 mL min^{-1}. The resolution between both enantiomers was greater than 3 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.

Keywords: 10-hydroxycamptothecin, (*R*)-enantiomer, validation, anti-cancer activity, quantification, HPLC

Introduction

10-Hydroxycamptothecin (Figure 1a) is a potent DNA *topoisomerase* I inhibitor. It induces apoptosis in human breast cancer cells. It inhibits the activity of *topoisomerase* I and has a broad spectrum of anti-cancer activity *in vitro* and *in vivo*. 10-HCTN is a single agent delivered by oral administration in the treatment of human colon cancer. 10-HCTN significantly repressed the proliferation of colon 205 cells at a relatively low concentration (5-20 nmol L^{-1}). 10-HCTN induced ultra-structural changes in nuclei and nuclear matrix were similar to those typically associated with lesions of DNA replication or RNA transcription.[1-4]

10-HCTN, a novel compound with an indole alkaloid structure, was clinically tested as a therapeutic agent by oral administration in the treatment of human colon cancer. The (*S*)-enantiomer has turned out to be a potent DNA topoisomerase I inhibitor.[5] No HPLC (high performance liquid chromatography) method is available in the literature for the chiral analysis of 10-HCTN.

10-HCTN is produced as a single isomer by a total synthesis and (*R*)-enantiomer could be present as a chiral impurity that is obtained in small amounts during the synthesis of 10-HCTN. Companies developing chiral

*e-mail: venkateshwarlu@avralab.com

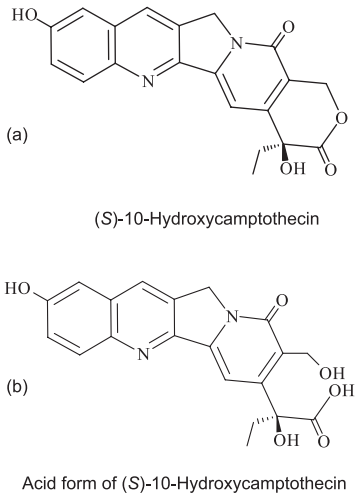

Figure 1. (a) Chemical structures of 10-HCTN and (b) hydrolyzed product (ring-opened form) of 10-HCTN.

drugs, where only one enantiomer is responsible for bioactivity, have to ensure that the process for their production is designed and optimized to minimize the formation or presence of the unwanted enantiomer to trace or below detection levels before taking the drug for toxicological, physical, pharmacokinetic and metabolic evaluation and determine its therapeutic benefits.[6,7] Such stringent levels of purity require sensitive and reliable analytical methods that allow for the detection of these unwanted isomers.[8] To the best of our knowledge, no HPLC method is available in the literature for the chiral analysis of 10-HCTN.[9,10]

Separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetics and different pharmacological or toxicological effects.[11,12]

In recent 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 extremely challenging due to the fact that enantiomers possess virtually identical properties.[13] 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 determine the enantiomers of an enantiomeric pair.[14,15] 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.

A simple and rapid isocratic LC method is often more preferred in ordinary lab. Polysaccharide chiral stationary phases are quite 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*)-10-HCTN. To the continuation of our reverse phase method development for 10-HCTN,[16] we initiated some work to develop a normal phase HPLC method for the quantitative determination of (*R*)-enantiomer in 10-HCTN. The present research work deals with rapid, simple, precise and robust enantioselective isocratic chiral LC method for the enantiomeric separation of 10-HCTN using an immobilized polysaccharide based chiral stationary phase (Chiralpak IC). This paper deals with the validation of determination of the (*R*)-enantiomer in 10-HCTN drug substance and drug product.

Experimental

Chemicals and reagents

10-HCTN was synthesized by a total synthesis. (*R*)-Enantiomer was prepared by using preparative HPLC in the laboratory. HPLC grade *n*-hexane was purchased 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 0.5 mg mL^{-1}.

Instrumentation

High performance liquid chromatography (HPLC)

A Shimadzu HPLC system LC-2010 CHT (Kyoto, Japan) 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 10-HCTN (0.5 mg mL^{-1}) and (*R*)-enantiomer (0.5 mg mL^{-1}) were prepared by dissolving an appropriate amount of ingredients in diluent (ethanol). For quantification of (*R*)-enantiomer in 10-HCTN, a solution of 0.5 μg mL^{-1} 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 mL min^{-1}. A wavelength of 270 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 different experiments based on nature and structure of compound. The design of mobile phase consists of a combination of alkane and polar alcohols based on normal or polar interactive modes. Initiated the screening analysis with the above combination of experiments to derive best suitable column and mobile phase conditions.

The racemic mixture was prepared by physical mixing of equal proportions of (*R*) and (*S*) 10-HCTN (0.5 mg of each sample). A 0.5 mg mL^{-1} 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 10-HCTN, different mobile phases were employed.

Several 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 10-HCTN. While using the chromatographic conditions like chiralcel OD(H) and chiralcel OD columns with flow rate 1.0 mL min^{-1} and mobile phase of *n*-hexane, isopropyl alcohol (50:50) (v/v) mixture, it has been observed that the enantiomers of 10-HCTN were eluted as broad peaks and the resolution (Rs) between isomers is very low. Inadequate 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 < 1.4) between 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 would provide considerable separation between the isomers with longer retention times. Further trials were 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 comparatively 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*) 10-HCTN and (*S*) 10-HCTN are eluted at 8.77 min and 10.4 min respectively (Figure 2a). The peak purity of (*S*) 10-HCTN is found to be homogeneous in all spiked samples. The resolution (Rs) between the two enantiomers was about 3.2. Diluent ethanol was used as blank and there was no interference of the blank with (*R*) and (*S*) isomers of 10-HCTN. 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 10-HCTN (*R*) and (*S*) isomers 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 of 270 nm. Then, the same conditions were maintained for the determination of (*R*) 10-HCTN in (*S*) 10-HCTN.

When 10-HCTN compound was subjected to base (1 N NaOH for 24 h at 60 °C), the lactone ring of 10-HCTN is open in an alkaline environment and the acid form of 10-HCTN (ring-opened form, Figure 1b) is analyzed using the same chiral normal phase method. The hydrolyzed product is clearly separated from the two enantiomers (Figure 2b). Hence there is no interference with the quantification of (*R*) and (*S*)-enantiomers.

System suitability

The system suitability 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 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 0.5 mg mL^{-1} solution containing 10-HCTN 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 0.125 µg mL^{-1} (limit of quantification (LOQ)) to 0.75 µg mL^{-1} (25% to 150%) of the permitted maximum level of the (*R*)-enantiomer (0.125, 0.250, 0.375, 0.50 and 0.750 µg mL^{-1}, i.e., 25% (LOQ), 50%, 75%, 100% and 150%) and prepared in ethanol from (*R*)-enantiomer stock solution.

Regression curve was obtained by plotting peak area *vs.* 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 and product

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

The study was carried out in triplicate at 0.05%, 0.1% and 0.15% of the 10-HCTN target analyte concentration. The recovery of (*R*)-enantiomer was calculated by determining recovery of the spiked amount of (*R*)-enantiomer in 10-HCTN.

Sensitivity (limit of detection and limit of quantification of (*R*)-enantiomer)

Limit of detection (LOD) and limit of quantification (LOQ) of (*R*)-enantiomer were achieved by injecting a series of dilute solutions of (*R*)-enantiomer.[17]

The sensitivity of the method was determined by establishing the limit of detection and limit of quantification for (*R*)-enantiomer, which was estimated using slope method (ICH Q2 (R1))[18] by injecting a series of dilute solutions of a known concentration.

The precision study was carried out 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 deliberately altered, and chromatographic resolution between enantiomers was evaluated.

The flow rate of the mobile phase was 1.0 mL min^{-1}. To study the effect of flow rate on the resolution, 0.1 units were changed from 0.9 to 1.1 mL min^{-1}. The effect of column temperature on resolution of both isomers was studied at 38 °C and 42 °C instead of 40 °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 10-HCTN in 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 evaluating the content of (*R*)-enantiomer in 10-HCTN. The same mobile phase was used for the 48 h during the 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.[19]

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 10-HCTN. In addition the dipole-dipole interaction occurs between the C=O group on the CSP and C=O group on the 10-HCTN.

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*)-10-HCTN. Another advantage of Chiralpak IC column is their greater stability under normal operation than other Daicel chiral columns. Immobilized column have good 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 10-HCTN was shown in Figure 2a. An excellent resolution (Rs = 3.2) between two enantiomers and ideal peak shape with tailing factor 1.18 was obtained. The system suitability test results of the chiral liquid chromatographic method on Chiralpak IC are presented in (Table 1).

Validation results

In the precision study, the percentage relative standard deviation (RSD) was less than 0.5% for the retention times of the enantiomers, 0.43% for 10-HCTN peak area and 0.41% for peak area of (*R*)-enantiomer. In the intermediate

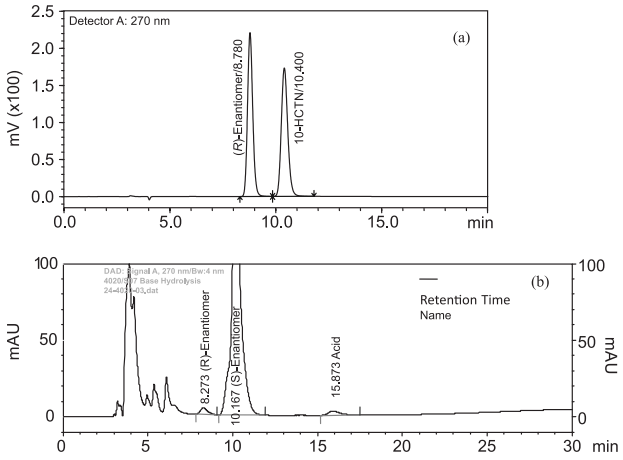

Figure 2. (a) Typical HPLC chromatogram of (*R*)-enantiomer and 10-HCTN (1:1) and (b) typical HPLC chromatogram of 10-HCTN compound when subjected to base (1 N NaOH for 24 h at 60 °C).

Table 1. Data of system suitability and specificity

Parameter	(*R*)-enantiomer	10-HCTN
Retention time / min	8.78	10.40
Resolution (Rs)	–	3.2
USP tailing factor (T)	1.16	1.18
No. of theoretical plates	6005	5569
Retention time RSD / %	0.11	–
Peak area RSD / %	0.10	–

USP: United States Pharmacopeia

precision study, the results showed that RSD values were in the same order of magnitude than those obtained for repeatability.

The LOD and LOQ concentration were estimated to be 0.04 and 0.13 µg mL^{-1} 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 1.0% RSD.

The described method was linear in the range of 0.125 to 0.75 µg mL^{-1} for (*R*)-enantiomer in 10-HCTN. The calibration curve was drawn by plotting the peak area of (*R*)-enantiomer *vs.* its corresponding concentration with correlation coefficient of 0.999 (Table 2). The equation of the calibration curve for (*R*)-enantiomer was y = 38170025.211x – 188.64 (Figure 3).

The addition and recovery experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 0.05, 0.10 and 0.15% of the analyte concentration. Percentage recovery ranged from 99.2 to 100.4%.

A HPLC chromatogram of (*R*)-enantiomer, of spiked (*R*)-enantiomer at 0.1% level in 10-HCTN sample and of 10-HCTN are shown in Figure 4.

The chromatographic resolution of the 10-HCTN and (*R*)-enantiomer peaks were used to assess the method

Table 2. Linearity data of (*R*)-enantiomer.

S. no.	Concentration / (mg mL^{-1})	*R*-isomer peak (n = 3)
1	0.000125	4425
2	0.000250	9825
3	0.000375	14473
4	0.00050	19400
5	0.000750	28953
Correlation coefficient	0.9998	
Slope	39009765.76	
Y-intercept	−188.6	
R^2	0.9995	

R^2: Coefficient of determination.

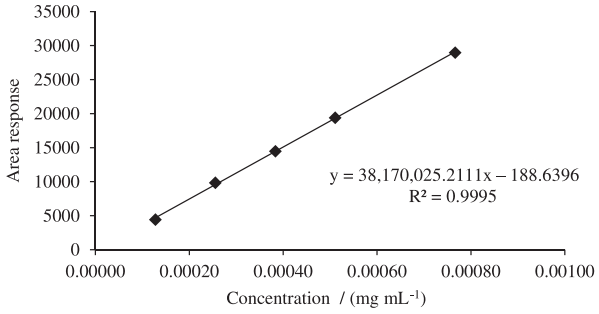

Figure 3. Linearity graph of (*R*)-enantiomer.

robustness under modified conditions. The resolution between 10-HCTN and (*R*)-enantiomer was greater than 3.0 under all separation conditions tested (Table 3), demonstrating sufficient robustness.

The RSD% of (*R*)-enantiomer content during solution stability and mobile phase stability experiments was below 0.5%. Hence 10-HCTN sample solution and mobile phase were stable for at least 48 h.

Analysis has been carried out on three different batches of 10-HCTN samples and observed *R*-isomer content is 0.07%, 0.08% and 0.08%.

Conclusions

A simple, rapid and accurate normal phase chiral HPLC method has been developed and validated for the enantiomeric separation of 10-HCTN. 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 shows right order of elution of (*R*)-enantiomer and (*S*)-enantiomer. The developed method is more suitable

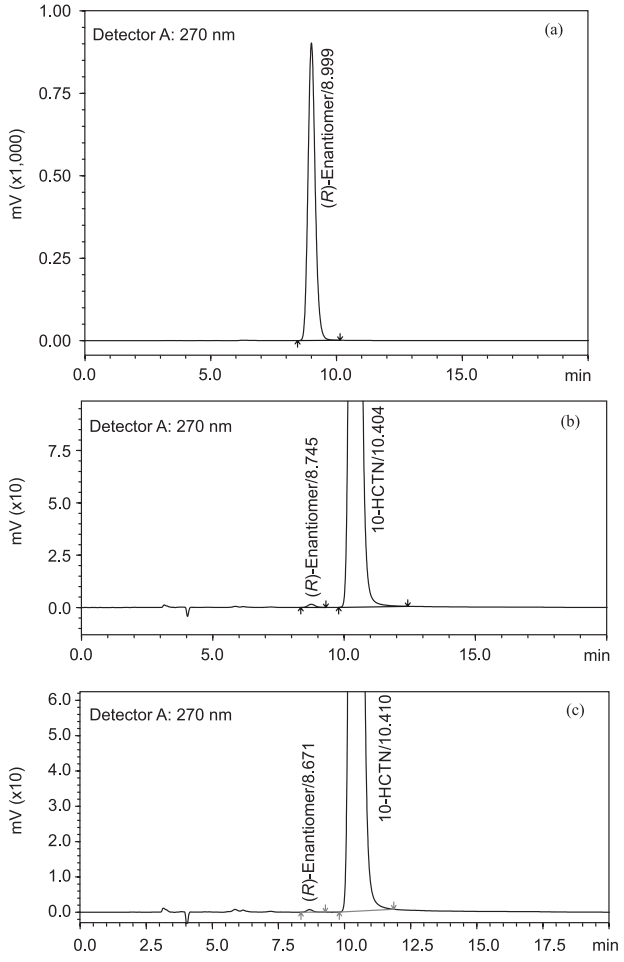

Figure 4. (a) Typical HPLC chromatogram of (*R*)-enantiomer; (b) typical HPLC chromatogram of 10-HCTN spiked with (*R*)-enantiomer at 0.1% specification level and (c) typical HPLC chromatogram of 10-HCTN sample.

Table 3. Data of robustness of the chiral LC method

Parameter	USP resolution between 10-HCTN and (*R*)-enantiomer
Column flow/ (mL min^{-1})	
0.9	3.32
1.0	3.20
1.1	3.07
Column temperature / °C	
38	3.04
40	3.19
42	3.25
Organic composition	
Hexane: IPA (50:50 v/v)	
55:45	3.39
50:50	3.19
45:55	3.05

with respective to resolution (> 3), number of theoretical plates (> 5000), USP tailing (< 1.1) and percentage recovery of the (*R*)-enantiomer between 99.2 to 100.4%.

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