



## A VALIDATED CHIRAL LC METHOD FOR THE ENANTIOMERIC SEPARATION OF TOPOTECAN HYDROCHLORIDE ON IMMOBILIZED AMYLOSE BASED STATIONARY PHASE

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### ABSTRACT

A simple, rapid, isocratic, normal phase chiral HPLC method was developed and validated for the enantiomeric separation of topotecan, (S)-10-[(dimethylamino)methyl]-4-ethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinoline-3,14(4H,12H)-dione monohydrochloride, an antineoplastic drug substance. The enantiomers of topotecan were resolved on a Chiralpak ADH (immobilized amylose based stationary phase) column using a mobile phase consisting of Methanol:acetonitrile:diethyl amine (70:30:0.2, v/v/v) at a flow rate of 1.0 mL min<sup>-1</sup>. The resolution between both enantiomers was greater than 2 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:** Topotecan, enantiomeric purity, chiral LC, method validation

### INTRODUCTION

Topotecan hydrochloride (trade name Hycamtin) is a chemotherapy agent that is a topoisomerase I inhibitor. It is the water-soluble derivative of camptothecin. It is used to treat ovarian cancer and lung cancer, as well as other cancer types. (S)-10-[(dimethylamino)methyl]-4-ethyl-4,9-dihydroxy-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinoline-3,14(4H,12H)-dione monohydrochloride After GlaxoSmithKline received final FDA approval for Hycamtin Capsules on October 15, 2007, Topotecan is the first topoisomerase inhibitor for oral use. Topotecan acts by forming a stable covalent complex with the DNA topoisomerase I aggregate, the so-called 'cleavable complex'. This process leads to breaks in the DNA strand resulting in apoptosis<sup>1</sup>. Hycamtin or topotecan is a semi-synthetic derivative of camptothecin. Camptothecin is a natural product extracted from the bark of the tree *Camptotheca acuminata*. Topoisomerase-I is a nuclear enzyme that relieves torsional strain in DNA by opening single strand breaks. Once topoisomerase-I creates a single strand break, the DNA can rotate in front of the advancing replication fork. Topotecan intercalates between DNA bases. This intercalation disrupts the DNA duplication machinery when it reaches a site where topotecan is intercalated. This disruption prevents DNA replication, and ultimately leads to cell death. Mammalian cells cannot efficiently repair these double strand breaks<sup>2</sup>. This process leads to breaks in the DNA strand resulting in apoptosis.

Topotecan is produced as a single isomer and (R)-enantiomer could be present as a chiral impurity. An HPLC method was reported in the literature for the enantiomeric separation of Topotecan using chiral AGP (protein based chiral stationary phase) column and Chiralpak IA (amylose based stationary phase) column. In the reported method enantiomeric separation achieved using isocratic elution. If the reported method is applied for the enantiomeric separation of topotecan saturation of column required about one and half hour and problem of base line drift occurred, the number of theoretical plates about 4000, peak tailing 1.5, broad peak shape and resolution about 1.85. In the reported method

resolution is more than 3, but the run time of analysis is more than 25 min and this article lacks of drug product analysis.

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.

In recent years, research has been intensified to understand the aspects of the molecular mechanism for stereo selective 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. 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. 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-based (amylose based) stationary phase are quite popular with wide recognition for direct resolution of enantiomers. The present research work deals with rapid, simple, precise and robust enantioselective isocratic chiral LC method for the enantiomeric separation of topotecan using an immobilized amylose based chiral stationary phase (Chiralpak ADH). This paper deals with the validation of determination of the (R)-enantiomer in topotecan drug substance and drug product.<sup>3</sup>

### EXPERIMENTAL

#### Chemicals

Samples of (R)-enantiomer and topotecan were obtained from Wockhardt Research Centre, Aurangabad, India. The drug product (HYCAMTIN) of topotecan label claims (topotecan 4 mg, Wockhardt Research Centre, Aurangabad, India) was purchased from the market. HPLC grade Methanol and acetonitrile was purchased from Qualigens Fine chemicals,

Mumbai, India. The HPLC grade diethyl amine were purchased from Merck Ltd, Mumbai, India.

#### Equipment

HPLC system used was an Waters, Empower (2649 series, USA), system equipped with auto sampler, quaternary pump, degasser and a UV detector. The output signal was monitored and processed using Empower software.

#### Sample preparation

The stock solution of (*R*) and (*S*)-enantiomers of topotecan (2.0 mg mL<sup>-1</sup>) was prepared by dissolving appropriate amount of substance in Methanol and Acetonitrile ( 50:40 v/v). For quantification of (*R*)-enantiomer in topotecan, a solution of 0.2 mg mL<sup>-1</sup> concentration was used.

#### Chromatographic condition

The chromatographic column used was Chiralpak ADH, 250 x 4.6 mm, 5 $\mu$ m (Daicel Chemical Industries, Ltd., Tokyo, Japan) . The mobile phase was Methanol : acetonitrile: diethyl amine (70:30:0.2, v/v/v). The flow rate of the mobile phase was 1.0 mL min<sup>-1</sup>. The column temperature was maintained at 30 °C and the eluent was monitored at a wavelength 267 nm. The injection volume was 10  $\mu$ L.

#### Method development

The racemic mixture was prepared by physical mixing of equal proportions of (*R*) and (*S*)- topotecan (0.2 mg of each sample). A 0.1 mg mL<sup>-1</sup> solution of racemic mixture was prepared in Methanol and Acetonitrile ( 50:40 v/v) diluent and used for the method development. To develop the suitable chiral HPLC method for the separation of the enantiomers of topotecan, different mobile phases were employed. Various experiments were carried out to select the best mobile phase that would give the optimum resolution and selectivity for the two enantiomers.

The enantiomeric separation for topotecan was not achieved by using n-hexane on Chiralpak IA column. There was an indication of separation on Chiralpak ADH column using the mobile phase consisting of Methanol:acetonitrile (70:30, v/v) but the peak shapes were slightly broad. For further improvement in resolution, peak shape and column efficiency, the peak modifier diethyl amine was used. Interesting observations was found when 0.2% diethyl amine was used in the mobile phase; both the peak shape and resolution were improved <sup>4</sup>. Very good separation was achieved on Chiralpak ADH column (resolution between enantiomers was found to be > 2) using the mobile phase Methanol : acetonitrile: diethyl amine (70:30:0.2, v/v/v).

#### METHOD VALIDATION

##### 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 2 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<sup>-1</sup> solution containing (*S*)-topotecan spiked with (*R*)-enantiomer (0.5%) was carried out. The intermediate precision was also

evaluated over three days by performing six successive injections each day. <sup>5</sup>

##### Linearity of (*R*)-enantiomer

Linearity was assessed by preparing six calibration sample solutions of (*R*)-enantiomer covering from 0.35  $\mu$ g mL<sup>-1</sup> (LOQ) to 3.75  $\mu$ g mL<sup>-1</sup> (0.35, 0.50, 0.80, 1.0, 1.20 and 1.50  $\mu$ g mL<sup>-1</sup>), prepared in diluent phase from (*R*)-enantiomer stock solution.

Regression curve was obtained 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.

##### 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 0.4, 0.5 and 0.6% of the (*S*)-topotecan target analyte concentration. The recovery of (*R*)-enantiomer was calculated by determining recovery of the spiked amount of (*R*)-enantiomer in topotecan.

##### Limit of detection and 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. The precision of the developed enantioselective method for (*R*)-enantiomer at limit of quantification was checked by analyzing six test solutions prepared at LOQ level and calculating the percentage relative standard deviation of area.

##### Robustness of the methods

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

The flow rate of the mobile phase was 1.0 mL min<sup>-1</sup>. To study the effect of flow rate on resolution of enantiomers, it was changed 0.1 units from 0.9 to 1.1 mL min<sup>-1</sup>. The effect of change in percent of ethanol on resolution was studied by varying from -1 to +1% while the other mobile phase components were held constant, as stated in chromatographic condition section. The effect of column temperature on resolution was studied at 28 °C and 32 °C instead of 30 °C while other mobile phase components were held constant, as stated in chromatographic condition section.

##### Solution stability and mobile phase stability

Stability of (*S*)- topotecan in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on laboratory bench for 3 days. Content of (*R*)-enantiomer was checked at 8 h intervals up to the study period.

Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in (*S*)-topotecan . For 3-days same mobile phase was used during the study period.

#### RESULTS AND DISCUSSION

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.<sup>6</sup>

The chiral stationary phase (CSP) that gave the best separation was Chiralpak ADH which is Amylose tris (3,5-dimethylphenylcarbamate) coated on 5µm silica-gel. The separation of enantiomers on Chiralpak ADH was due to the interaction between the solute and the polar phenylcarbamate group on the CSP. In addition the dipole-dipole interaction occurs between the C=O group on the CSP and C=O group on the topotecan.<sup>7-8</sup>

Topotecan is an acidic compound and to prevent peak tailing from the presence of extra high activity, small amount of diethyl amine was used in the mobile phase, to help block active sites and improve peak symmetry. Introduction of diethyl amine in the mobile phase enhanced the chromatographic efficiency, peak symmetry and resolution between the enantiomers. It played an important role for the improvement of resolution, column efficiency and peak shape.

The immobilized amylose-based stationary phase in Chiralpak ADH column has higher selectivity than protein based (chiral AGP) and amylose based (Chiralpak IA) columns, being suitable for the enantioselective separation and accurate quantification of (R)-topotecan. Another advantage of Chiralpak ADH column is their greater stability under normal operation than other Daicel chiral columns. Immobilized column have good stability to strong solvents like THF, ethyl acetate, and chlorinated solvents. Using immobilized stationary phase columns allows a great freedom of solvent choices.<sup>9</sup>

A representative chromatogram of the enantiomeric resolution of topotecan was shown in (Figure 2-3). An excellent resolution ( $R_s = 5.68$ ) 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 ADH 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, 0.27% for (S)-topotecan peak area and 0.29% for (R)-enantiomer peak area. In the intermediate precision study, the results showed that R.S.D. values were in the same order of magnitude than those obtained for repeatability.

The limit of detection (LOD) and limit of quantification (LOQ) concentration were estimated to be 0.22 and 0.35 µg mL<sup>-1</sup> 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 1.5 RSD%. The described method was linear in the range of 0.35 to 1.50 µg mL<sup>-1</sup> for (R)-enantiomer in topotecan. The calibration curve was drawn by plotting the peak area of (R)-enantiomer versus its corresponding concentration with correlation coefficient of 0.9998. The equation of the calibration curve for (R)-enantiomer was  $Y = 3.9436x - 1.1712$ . The percentage relative standard deviation of the slope and Y-intercept of the calibration curve were 1.2 and 1.5 respectively.<sup>10-11</sup>

The recovery and standard addition experiments were conducted for (R) enantiomer in bulk samples in triplicate at 50, 100 and 150 % of the analyte concentration. Percentage recovery ranged from 98.0 to 101.0%.

A HPLC chromatogram of (R)-enantiomer is shown in Figure 3, a HPLC chromatogram of topotecan tablet is shown in Table 2.

The chromatographic resolution of the (S)- topotecan and (R)-enantiomer peaks was used to evaluate the method robustness under modified conditions. The resolution between (S)- topotecan and (R)-enantiomer was greater than 2.0 under all separation conditions tested (Table 2), demonstrating sufficient robustness.

#### CONCLUSION

A simple, rapid and accurate normal phase chiral HPLC method has been developed and validated for the enantiomeric separation of repaglinide. Chiralpak IA (immobilized amylose-based chiral stationary phase) was found to be selective for the enantiomers of the drug. The method was completely validated 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 than reported methods. The developed method shows right order of elution of (R)-enantiomer and (S)-enantiomer. The developed method is more suitable than the reported methods with respective to resolution ( $> 2$ ), number of theoretical plates ( $> 5000$ ), USP tailing ( $< 1.3$ ), the percentage recovery of the (R)-enantiomer between 98.5 to 101.5%.

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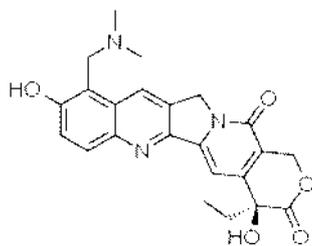
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**Table 1: System suitability results**

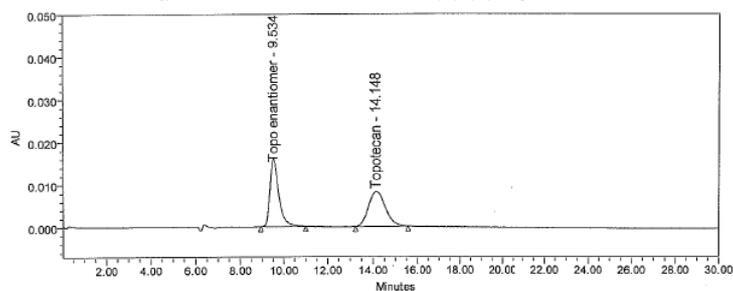
Parameter	R	S
Retention time / min	9.5	14.1
Resolution ( $R_s$ )		5.68
USP Tailing	1.22	1.18
Theoretical plates	6112	5879
% RSD retention time	0.29	0.27
% RSD peak area	0.7	0.6

**Table 2: Robustness of the chiral LC method**

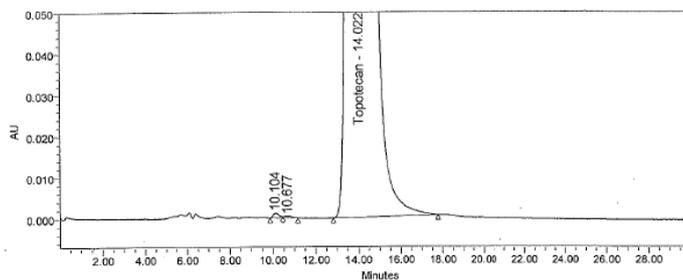
Parameter	Resolution between repaglinide and (R)-enantiomer
Flow rate / (mL min <sup>-1</sup> )	
0.9	5.22
1.0	5.09
1.1	5.11
Column Temperature / oC	
28	4.99
30	5.02
32	5.12
Acetonitrile percentage in the mobile phase / %	
29	4.98
30	5.23
31	5.14



**Figure 1: Chemical structure of (+)-(-S)-topotecan**



**Figure 2: A chromatogram of the Topotecan Hydrochloride system suitability solution.**



**Figure 3: A chromatogram of the Topotecan Hydrochloride Sample preparation**