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Short communication

# Development and validation of enantioselective high performance liquid chromatographic method for Valacyclovir, an antiviral drug in drug substance

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**Abstract**

A chiral high performance liquid chromatographic method was developed and validated for the enantiomeric resolution of Valacyclovir, L-valine 2-[(2-amino-1,6-dihydro-6-oxo-9h-purin-9-yl) methoxy] ethyl ester, an antiviral agent in bulk drug substance. The enantiomers of Valacyclovir were resolved on a Chiralpak AD (250 mm × 4.6 mm, 10 μm) column using a mobile phase system containing *n*-hexane: ethanol: diethylamine (30:70:0.1, v/v/v). The resolution between the enantiomers was found not less than four. The presence of diethylamine in the mobile phase has played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection and limit of quantification of (D)-enantiomer were found to be 300 and 900 ng/ml, respectively, for 20 μL injection volume. The calibration curve showed excellent linearity over the concentration range of 900 ng/ml (LOQ) to 6000 ng/ml for (D)-enantiomer. The percentage recovery of (D)-enantiomer was ranged from 97.50 to 102.18 in bulk drug samples of Valacyclovir. Valacyclovir sample solution and mobile phase were found to be stable for at least 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (D)-enantiomer in bulk drugs substance. It can be also used to test the stability samples of Valacyclovir.
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*Keywords:* Valacyclovir; An antiviral agent; Chiral HPLC; Validation and enantiomeric resolution

## 1. Introduction

Valacyclovir is L-valyl ester pro-drug of the antiviral drug acyclovir. The chemical name of Valacyclovir is L-valine 2-[(2-amino-1,6-dihydro-6-oxo-9h-purin-9-yl) methoxy] ethyl ester. Acyclovir is a specific and selective inhibitor of viral herpes replication and is neither highly lipid nor aqueous soluble, with limited and variable oral bioavailability (15–21%) that decreases with increasing doses. Conversely, Valacyclovir has an oral bioavailability of three to five times higher than that of acyclovir itself. Moreover, Valacyclovir is rapidly and extensively hydrolyzed to acyclovir after oral administration. Studies of its transport mechanisms have demonstrated that the improved oral bioavailability is due to its active transport by human intestinal peptide transporter (PEPT1). Valacyclovir hydrochloride is rapidly converted to acyclovir, which has demonstrated antiviral activity against herpes simplex virus types, 1 (HSV-1) and 2 (HSV-2) and varicella-zoster virus (VZV) both in vitro and in vivo. The inhibitory activity of acyclovir is highly selective due to its affinity for the enzymes thymidine kinase (TK) encoded by HSV and VZV. Neither Valacyclovir nor acyclovir is metabolized by cytochrome P450 enzymes [1,2].

Degradation and penetration of the antiviral drugs: the use of pro-drugs might improve drug absorption and other pharmacokinetics properties. Valacyclovir, a valyl ester of acyclovir is better absorbed into the gastrointestinal tract than the parent drug, and is cleaved by host enzymes into valine (a natural amino acid) and acyclovir [3].

Few HPLC methods were reported in the literature for the quantitative determination of Valacyclovir in tablets, human serum and human biological fluids [4–6].

Valacyclovir is produced as a single isomer and that of the (D)-isomer could be present as a chiral impurity. In the literature, there is no reference for the enantiomeric separation of Valacyclovir in bulk drugs using high performance liquid chromatography.

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Enantiomers of racemic drugs often differ in pharmacokinetic behaviour or pharmacological action [7].

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 [8]. Recently, much work has been reported describing the use of chiral stationary phases, in conjunction with HPLC, as a way to separate and thereby individually quantitate the enantiomers of an enantiomeric pair [9–11].

This article describes normal phase high performance liquid chromatographic method for the enantiomeric separation of Valacyclovir using an amylose based chiral stationary phase, Chiralpak AD. The developed normal phase high performance liquid chromatographic method was validated for determination of (D)-enantiomer in L-Valacyclovir bulk sample.

## 2. Experimental

### 2.1. Chemicals

(L) and (D) enantiomers of Valacyclovir were synthesized and kindly supplied by Process Research Department of Wockhardt Limited, Mumbai, India and the chemical structure was given in Fig. 1. HPLC grade *n*-hexane and ethanol were purchased from Merck, Germany. Laboratory reagent grade diethylamine was purchased from Qualigens Fine Chemicals, Mumbai, India.

### 2.2. Equipment

A Shimadzu 2010 series LC system with photo diode array detector and inbuilt auto injector (Shimadzu Crop., Kyoto, Japan.) was utilized for method development and validation. The output signal was monitored and processed using LC Solution software (Shimadzu Crop., Kyoto, Japan) on Pentium computer (Digital Equipment Co.).

### 2.3. Sample preparation

Stock solutions of (D)-enantiomer (100 μg/ml) and (L) Valacyclovir (5 mg/ml) were prepared by dissolving the appropriate amount of the substances in ethanol. The analyte concentration of Valacyclovir was fixed as 1.0 mg/ml. Working solutions of L-Valacyclovir and (D)-enantiomer was prepared in mobile phase.

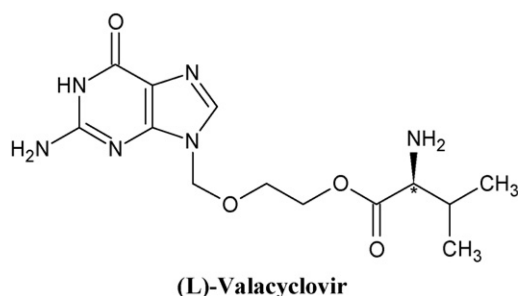


Fig. 1. Chemical structure of (L)-Valacyclovir.

### 2.4. Chromatographic conditions

The chromatographic conditions were optimized using an amylose based chiral stationary phase Chiralpak AD (250 mm × 4.6 mm, 10 μm, Daicel Chemical Industries, Ltd., Tokyo, Japan) which was safeguarded with a 1 cm long guard column. The mobile phase was *n*-hexane:ethanol:diethylamine (30:70:0.1, v/v/v). The flow rate was set at 1.0 ml/min. The column was maintained at 25 °C and the detection was carried out at a wavelength of 254 nm. The run time was set to 20 min. The injection volume was 20 μl. Protein based chiral stationary phase Chiral AGP (Chrom Tech Ltd., Cheshire, UK), Cellulose based chiral stationary phase Chiralcel OJ-H (Diacel Chemical Industries, Ltd., Tokyo, Japan), and Pirkle based chiral stationary phase Whelk-O-1 (Merck KgaA, Darmstadt, Germany) were also employed during method development.

### 2.5. Validation of the method

#### 2.5.1. Method reproducibility

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 1.0 mg/ml solution containing (L)-Valacyclovir spiked with (D)-enantiomer (0.5%) was carried out. The intermediate precision was also evaluated over three days by performing six successive injections each day.

#### 2.5.2. Limit of detection and limit of quantification of (D)-enantiomer

LOD and LOQ [12] were achieved by injecting a series of dilute solutions of (D)-enantiomer.

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

#### 2.5.3. Linearity of (D)-enantiomer

Detector response linearity was assessed by preparing six calibration sample solutions of (D)-enantiomer covering from 900 ng/ml (LOQ) to 6000 ng/ml (900, 1800, 2700, 3600, 4500 and 6000 ng/ml), prepared in mobile phase from (D)-enantiomer stock solution.

Regression curve was obtained by plotting peak area versus concentration, using the least squares method. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and *Y*-intercept of the calibration curve was calculated.

#### 2.5.4. Quantification of (D)-enantiomer in bulk sample

The L-Valacyclovir bulk sample, provided by Process Research Department of Wockhardt Limited, does not show the presence of (D)-enantiomer. Standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantification of (D)-enantiomer in bulk drug samples.

The study was carried out in triplicate at 0.4, 0.5 and 0.6% of the L-Valacyclovir target analyte concentration. The recovery of (D)-enantiomer was calculated from the slope and *Y*-intercept of the calibration curve obtained.

#### 2.5.5. Robustness

To determine robustness of the method, experimental conditions were purposely altered and chromatographic resolution between (L)-Valacyclovir and (D)-enantiomer was evaluated.

The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution of enantiomers, 0.2 units changed it from 0.8 to 1.2 ml/min. The effect of change in percent ethanol on resolution was studied by varying from −1 to +1% while the other mobile phase components were held constant as stated in Section 2.4. The effect of column temperature on resolution was studied at 20 and 30 °C instead of 25 °C while the other mobile phase components were held constant as stated in Section 2.4.

#### 2.5.6. Solution stability and mobile phase stability

Stability of L-Valacyclovir in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for two days. Content of (D)-enantiomer was checked for 6 h interval up to the study period.

Mobile phase stability was carried out by evaluating the content of (D)-enantiomer in L-Valacyclovir sample solutions prepared freshly at 6 h interval for 2 days. Same mobile phase was used during the study period.

## 3. Results and discussion

### 3.1. Optimization of chromatographic conditions

The aim of this work is to separate the enantiomers of Valacyclovir and accurate quantification of (D)-enantiomer. Recemic mixture solution of 0.5 mg/ml prepared in mobile phase was used in the method development. To develop a rugged and suitable liquid chromatographic method for the separation of Valacyclovir enantiomers, different mobile phases and stationary phases were employed. The main target of the liquid chromatographic method is to get the separation the enantiomers of Valacyclovir, various chiral columns and various experiments were conducted, to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. No separation was found on Chiralcel OJ-H, Whelk-O-1 and Chiralcel AGP columns using different possible mobile phases. There is an indication of separation on Chiralpak AD columns using a mobile phase consisting of *n*-hexane:ethanol (50:50, v/v) and the peak shapes were broad. Valacyclovir is a basic compound. To prevent peak tailing that may result from the presence of sites of extra high activity, small amount of organic base, diethylamine used in the mobile phase that may help to block any active sites and improve peak symmetry. Introduction of diethylamine in the mobile phase enhanced the chromatographic efficiency, peak symmetry and resolution between the enantiomers. Very good separation was achieved on Chiralpak AD column (Resolution between enantiomers was found greater than four) using the mobile phase system *n*-hexane:ethanol:diethylamine (30:70:0.1, v/v/v). Valacyclovir having only one chiral center. Amylose forms a helical structure. Helical amylose derivative contains five chiral centers. As a result the polymers contain a large number of chirally active sites and thus a relatively high probability of chiral site interaction with the solute. It could be due to high probability of interaction, better resolution was found on Chiralpak AD column. Due to the better chromatographic results

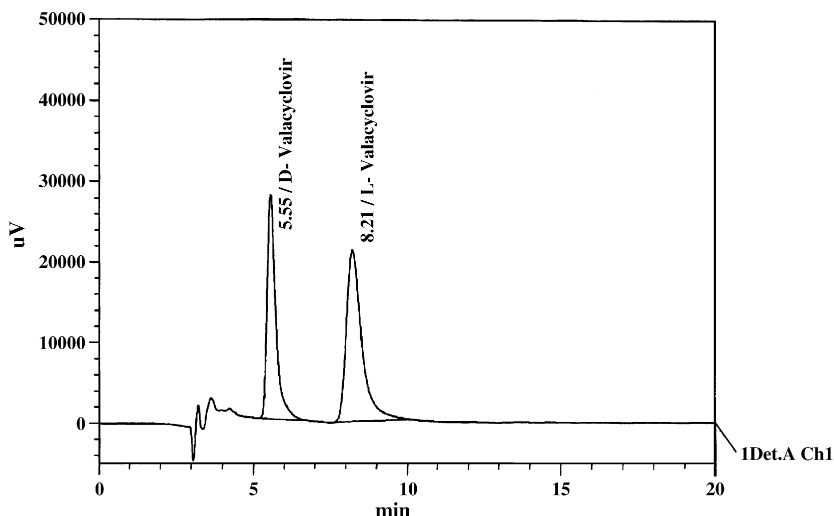


Fig. 2. Enantiomeric resolution of Valacyclovir on Chiralpak AD column. Mobile phase consisted of *n*-hexane:ethanol:diethylamine (30:70:0.1, v/v/v). Flow rate: 1.0 ml/min, UV-254 nm; column temperature: 25 °C.

Table 1
System-suitability report

| Compound ($n = 3$) | $R_t$ (min) | $R_s$ | $N$ | $T$ |
|---|---|---|---|---|
| (D)-Enantiomer | 5.5 | | 3357 | 1.6 |
| Valacyclovir | 8.1 | 4.1 | 3023 | 1.6 |

$n = 3$ determinations; $R_t$: retention time; $R_s$: USP resolution; $N$: number of theoretical plates (USP tangent method); $T$: USP tailing factor.

obtained on the Chiralpak AD column, the method validation was carried out on the same.

In the optimized method, the typical retention times of (D)-enantiomer and L-Valacyclovir were about 5.5 and 8.1 min, respectively. The enantiomeic separation of Valacyclovir on Chiralpak AD column was shown in Fig. 2. The system suitability test results of the chiral liquid chromatographic method on Chiralpak AD are presented in Table 1.

### 3.2. Validation results of the method

In the repeatability study, the relative standard deviation (R.S.D.) was less than 0.5% for the retention times of both the enantiomers, 0.9% for L-Valacyclovir peak area and 1.5% for (D)-enantiomer peak area (Table 2). In the intermediate precision study, results show that R.S.D. values were in the same order of magnitude than those obtained for repeatability (Table 2).

The limit of detection (LOD) and limit of quantification (LOQ) concentrations were estimated to be 300 and 900 ng/ml for (D)-enantiomer, when a signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for (D)-enantiomer at limit of quantification was less than 3% R.S.D. (Table 2).

Good linearity was observed for (D)-enantiomer over the concentration range of 900–6000 ng/ml, with the linear regression equation $y = 22.21533X - 1469.31441$ (correlation coefficient $R^2 = 0.99815$). Linearity was checked for (D)-enantiomer over the same concentration range for three consecutive days. The percentage relative standard deviation of the slope and $Y$-intercept of the calibration curve were 1.9 and 9, respectively (Table 2).

Table 2
Validation results of the developed enantioselective liquid chromatographic method

| Validation parameter | Results |
|---|---|
| Repeatability ($n = 6$, %R.S.D.) | |
| Retention time (D-enantiomer) | 0.4 |
| Retention time (L-enantiomer) | 0.4 |
| Peak area (D-enantiomer) | 1.5 |
| Peak area (L-enantiomer) | 0.9 |
| Intermediate precision ($n = 18$, %R.S.D.) | |
| Retention time (D-enantiomer) | 0.8 |
| Retention time (L-enantiomer) | 0.7 |
| Peak area (D-enantiomer) | 1.9 |
| Peak area (L-enantiomer) | 1.3 |
| LOD–LOQ (D-enantiomer) | |
| Limit of detection (ng/ml) | 300 |
| Limit of quantification (ng/ml) | 900 |
| Precision at LOQ (%R.S.D.) | 2.5 |
| Linearity (D-enantiomer) | |
| Calibration range (ng/ml) | 900–6000 |
| Calibration points | 6 |
| Correlation coefficient | 0.99815 |
| Slope (%R.S.D.) | 1.9 |
| Intercept (%R.S.D.) | 9 |

Table 3
Recovery results of (D)-enantiomer in bulk drugs

| Added (ng) ($n = 3$) | Recovered (ng) | % Recovery | %R.S.D. |
|---|---|---|---|
| 4002 | 3974 | 99.30 | 2.3 |
| 5003 | 5112 | 102.18 | 2.5 |
| 6005 | 5855 | 97.50 | 2.9 |

$n = 3$ determinations.

The standard addition and recovery experiments were conducted for (D)-enantiomer in bulk samples in triplicate at 0.4, 0.5 and 0.6% of analyte concentration. Recovery was calculated from slope and $Y$-intercept of the calibration curve obtained in linearity study and percentage recovery was ranged from 97.50 to 102.18 (Table 3).

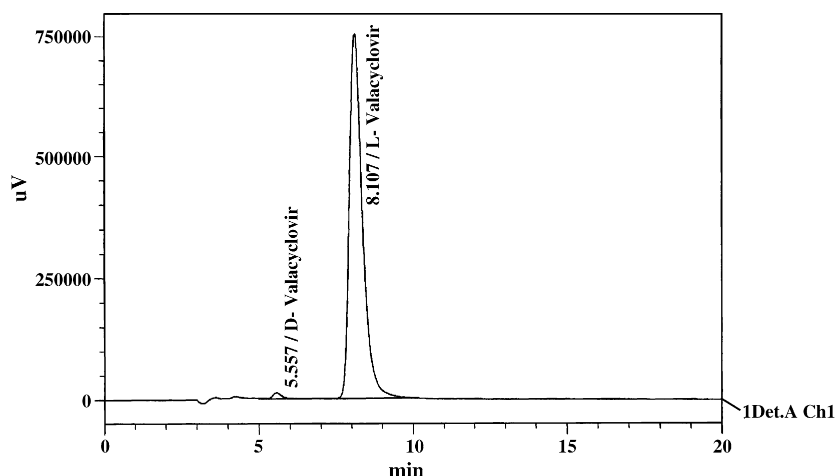


Fig. 3. Typical HPLC chromatogram of (L)-Valacyclovir bulk sample (1.0 mg/ml) spiked with (D)-enantiomer (0.5%).

Table 4
Robustness of the chiral liquid chromatographic method

| Parameter | USP resolution between Valacyclovir and (D)-enantiomer |
|---|---|
| Flow rate (ml/min) | |
| 0.8 | 4.3 |
| 1.0 | 4.1 |
| 1.2 | 3.9 |
| Column temperature (°C) | |
| 20 | 4.2 |
| 25 | 4.1 |
| 30 | 3.8 |
| Ethanol percentage in mobile phase | |
| 69 | 4.3 |
| 70 | 4.2 |
| 71 | 4.0 |

A HPLC chromatogram of spiked (D)-enantiomer at 0.5% level in L-Valacyclovir sample was shown in Fig. 3.

The chromatographic resolution between (L)-Valacyclovir and (D)-enantiomer peaks was used to evaluate the method robustness under modified conditions. The resolution between L-Valacyclovir and (D)-enantiomer was greater than 3.5, under all separation conditions tested (Table 4), demonstrating sufficient robustness.

The % R.S.D. of L-Valacyclovir content during solution stability and mobile phase stability experiments was within 1.5% R.S.D. Hence Valacyclovir sample solution and mobile phase were stable for at least 48 h.

## 4. Conclusion

A simple, rapid and accurate normal phase enantioselective high performance liquid chromatographic method was described for the enantiomeric separation of Valacyclovir. Amylose based chiral columns Chiralpak AD column found to be selective for the enantiomers of Valacyclovir. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be conveniently used by quality control department for the quantitative determination of chiral impurity (D-enantiomer) in bulk materials and also stability samples.

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