A Validated LC Method for the Determination of Chiral Purity of (S)-2-azido-3-methylbutanoic acid: A key Raw material of Valganciclovir hydrochloride

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ABSTRACT

A simple and accurate normal phase liquid chromatographic method was developed for the determination of chiral purity of (S)-2-azido-3-methylbutanoic acid, S-enantiomer used as key starting raw material in the manufacturing of valganciclovir hydrochloride bulk drug. Chromatographic separation between (S)-2-azido-3-methylbutanoic acid and its opposite enantiomer (R)-2-azido-3-methylbutanoic acid, R-enantiomer was achieved using a Chiralpak IA column using a mobile phase containing n-hexane, ethanol, isopropyl alcohol and tri-fluoro acetic acid (98:1.5:0.5:0.1 v/v/v/v). The resolution between the two enantiomers was found to be more than 2.0. The limit of detection (LOD) and limit of quantification (LOQ) of the R-enantiomer was 0.15 and 0.5 µg mL⁻¹, respectively, for 10 µL injection volume. The percentage recoveries of the R-enantiomer ranged from 96.5 to 105.3 in the samples of (S)-2-azido-3-methylbutanoic acid. The test solution and mobile phase was observed to be stable up to 24 h after the preparation. The developed method was validated as per International Conference on Harmonization guidelines in terms of LOD, LOQ, precision, linearity, accuracy, robustness and ruggedness.

Key words: Valganciclovir hydrochloride Enantiomeric separation Chiral Liquid chromatography Validation and Quantification.

INTRODUCTION

Most of the pharmaceutical industries are now concentrating towards the study of the therapeutic effects of enantiomers of the existing drug molecules. A control and accurate quantification of
undesired enantiomers in pharmaceuticals is essential [1] in this connection and LC is generally opted for this purpose. Valganciclovir hydrochloride [2] (Valcyte, manufactured by Roche) is an antiviral medication used to treat cytomegalovirus infections and hydrochloride salt of an ester of L-valine with ganciclovir that exists as a mixture of two diastereomers [3]. After oral administration [4], it is rapidly converted to ganciclovir by intestinal and hepatic esterases. (S)-2-azido-3-methylbutanoic acid, a single enantiomer starting raw material is very critical as it affects the overall chiral purity of valganciclovir hydrochloride bulk drug. To our present knowledge no chiral LC methods were reported in the literature for the chiral purity determination of (S)-2-azido-3-methylbutanoic acid. Therefore, it is felt necessary to develop a chiral LC method for the accurate quantification of the undesired (R)-enantiomer.

The present research work focused on the development of a chiral LC method for the determination of the enantiomeric purity of (S)-2-azido-3-methylbutanoic acid and to quantify the undesired (R)-enantiomer using various chiral LC columns. Very good resolution between S and R-enantiomers was observed on Chiralpak IA column. In the developed method, the S and R-enantiomers were well resolved with a resolution >2.0 within a 20 min run time using a simple normal phase system containing n-hexane, ethanol, isopropyl alcohol (IPA) and tri-fluoro acetic acid (TFA). This paper deals with the method development and validation of the developed method [5-7].

The determination of the stereo isomeric composition of pharmaceuticals is rapidly becoming one of the key issues in the development of new drugs. Among the methods currently used to achieve chiral separation of enantiomers, high resolution liquid chromatography systems based on chiral stationary phases (CSPs) are more rapid and are suitable for the resolution of racemic mixtures of pharmacologically active chemical entities [8-9].

**EXPERIMENTAL SECTION**

**Chemicals and Reagents**

Samples of S and R-enantiomers of 2-azido-3-methylbutanoic acid confirmed by spectral characterization and SOR (specific optical rotation) were obtained from Process Research Department of Dr. Reddy’s Laboratories Ltd, Hyderabad, India. HPLC-grade n-hexane was procured from Tedia company, Inc., Canada. Ethanol and IPA were purchased from Ranbaxy Fine Chemicals, New Delhi, India. Analytical Reagent grade tri-fluoroacetic acid (TFA) was purchased from Fluka.

**Instrumentation**

Waters make An Alliance HPLC (Alliance 2695 Model, Waters Corporation, Milford, USA) equipped with in-built auto sampler and 2487 dual λ absorbance detector was used for the analysis. The output signal was monitored and processed using Waters Millenium software. Photo diode array detection was also used for determining peak purity.

The chiral columns used in method development were Chiralcel OD, Chiralpak AD, Chiralcel OJ, Chiralpak IA, Chiralpak IB and Chiralpak IC [10]. All are Daicel make (Daicel Chemical Industries, Japan) with 5 µm particle size in (250 x 4.6) mm dimension. The other columns Crownpak (Daicel Chemical Industries, Japan), Chiral AGP (Advanced Separation Technologies
Inc., New Jersey) and Chirobiotic (Chiron Technologies, Inc., New Jersey) are 5 µm particle size in (150 x 4.6) mm dimension.

**Chromatographic Conditions**

The chromatographic conditions were optimized using a Chiralpak IA column. The mobile phase, a mixture of n-hexane, ethanol, isopropyl alcohol and TFA in the ratio of 98:1.5:0.5:0.1 mL with a flow rate of 1.0 mL min\(^{-1}\) was employed. The column temperature was maintained at 25 °C and the detection was monitored at a wavelength of 215 nm. The injection volume was 10 µL.

**Preparation of Standard Solutions**

The stock solutions of \(S\) and \(R\)-enantiomers of 2-azido-3-methylbutanoic acid were prepared individually by dissolving an appropriate amount of the substances in diluent of mobile phase. Working solutions were prepared in mobile phase. The target analyte concentration was fixed as 2.0 mg mL\(^{-1}\).

**RESULTS AND DISCUSSION**

**Method Development**

The objective of this work was to evaluate the enantiomeric purity of the \(S\)-enantiomer of 2-azido-3-methylbutanoic acid and accurate quantification of the undesired \(R\)-enantiomer. The preliminary trails carried out in reverse phase chiral columns were not fruitful in the separation of these isomers. Different chiral stationary phases were employed during the method development namely Chiralpak IA, Chiralpak IB, Chiralpak IC, Chiralpak AD-H, Chiralcel OD-H, Crownpak CR (+), Chiral AGP and Chirobiotic T. Different trials were made during the method development and details are mentioned in the Table 1.

Chiralpak IA, an amylose based chiral stationary phase was found to be selective for the enantiomers of 2-azido-3-methylbutanoic acid. Very good resolution was achieved on Chiralpak IA column using mobile phase contains the mixture of n-hexane, ethanol, isopropyl alcohol and TFA (98:1.5:0.5:0.1 v/v/v/v). The addition of IPA and TFA to the mobile phase plays an important role on enhancing the chromatographic efficiency and resolution between the enantiomers.

**Optimized Chromatographic Conditions**

Chromatographic base to base separation was achieved only on a Chiralpak IA (250 x 4.6 mm, 5 microns particle size) chiral column using the mobile phase, which contains the mixture of n-hexane, ethanol, isopropyl alcohol and TFA (98:1.5:0.5:0.1 v/v/v/v). The flow rate of the mobile phase was 1.0 mL min\(^{-1}\). The column temperature was maintained at 25°C and the detection wavelength was 215 nm. The injection volume was 10 µL. The total analysis time for each run was 20 min. Very good separation was observed within short runtime on Chiralpak IA column (resolution >2.0). The typical retention times of \(S\) and \(R\)-enantiomers of 2-azido-3-methylbutanoic acid are 7.7 and 8.4. The USP tailing factor (T) was found to be 1.2 for both \(S\) and \(R\)-enantiomers of 2-azido-3-methylbutanoic acid. The system suitability results were given in Table 1.
The structure and configurations of $R$ and $S$-isomers of 2-azido-3-methylbutanoic acid are displayed in Fig. 1. The chromatogram of the $S$-isomer spiked with $R$-enantiomer is displayed in Fig. 2.

**Method Validation**

**Precision**

The precision of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogenous sample under prescribed conditions. The system and method precision for the $R$-enantiomer were checked at its specification level (i.e. 0.5% with respect to analyte concentration, 2.0 mg mL$^{-1}$). The percentage RSD of method repeatability and system repeatability for the $R$-enantiomer were found to be 6.3 and 4.1, respectively, which confirms good precision of the method.

**Linearity**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. The linearity of the method for the $R$-enantiomer was checked at six concentration levels, i.e. from limit of quantitation (LOQ) (0.05%) to 200% of the undesired $R$-enantiomer specification level (0.5%), which is with respect to of (S)-2-azido-3-methylbutanoic acid analyte concentration. The coefficient of regression of the calibration curve was found to be 0.999, thus confirming the excellent correlation between the peak area and concentration of the $R$-enantiomer.

**Limit of Detection and Limit of Quantitation**

The limit of detection (LOD) represents the concentration of analyte that would yield a signal-to-noise ratio of 3 [11]. The limit of detection for the $R$-enantiomer was found to be 0.15 µg mL$^{-1}$ for 10 µL of injection volume. The LOQ represents the concentration of analyte that would yield a signal-to-noise ratio of 10 [11]. The limit of quantitation for the $R$-enantiomer was found to be 0.5 µg mL$^{-1}$ for the 10 µL of injection volume.

**Ruggedness and Robustness**

The ruggedness [11] of a method was defined as degree of reproducibility of results obtained by analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments and different days. The standard addition and recovery experiments carried out for the $R$-enantiomer in (S)-2-azido-3-methylbutanoic acid bulk samples at the same concentration levels tested in Laboratory A were again carried out at laboratory B using a different instrument and analyst. The data obtained from Laboratory B was well in agreement with the results obtained in Laboratory A, thus proving the method ruggedness. The robustness [11] of an analytical procedure is measured by its capability to remain unaffected through small, but deliberate, variations in method parameters and provide an indication of its reliability during normal usage. In the varied chromatographic conditions like flow rate, mobile phase ratio and column temperature, the resolution between the peaks of $S$ and $R$-enantiomers of 2-azido-3-methylbutanoic acid was found to be $>2.0$ illustrating the robustness of the method.
Quantitation of (R)-Enantiomer

Standard addition and recovery experiments were conducted to determine the accuracy of the present method, for the quantification of the (R)-enantiomer in samples of (S)-2-azido-3-methylbutanoic acid. The study was carried out at 0.25, 0.50 and 0.75% of target analyte concentration of (S)-2-azido-3-methylbutanoic acid. The percentage recoveries of the R-enantiomer ranged from 96.5 to 105.3 in samples of (S)-2-azido-3-methylbutanoic acid.

Table 1. Results of various trials and system suitability test.

<table>
<thead>
<tr>
<th>Trail no.</th>
<th>LC conditions</th>
<th>Retention factor (k)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Column: chiralpak-IA 250 x 4.6 mm, 5 µm, Mobile phase: n-hexane, ethanol, IPA, TFA (98:1.5:0.5:0.1, v/v/v/v), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 7.5 R-enantiomer: 8.1</td>
<td>Resolution between the pair of enantiomers: &gt; 2.0</td>
</tr>
<tr>
<td>2.</td>
<td>Column: chiralpak-IB 250 x 4.6 mm, 5 µm, Mobile phase: n-hexane, IPA, TFA (98:2:0.1, v/v/v), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 6.3 R-enantiomer: 6.3</td>
<td>S and R- enantiomers are co-eluted</td>
</tr>
<tr>
<td>3.</td>
<td>Column: chiralpak-IC 250 x 4.6 mm, 5 µm, Mobile phase: n-hexane, IPA, TFA (98:2:0.1, v/v/v), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 5.6 R-enantiomer: 5.6</td>
<td>S and R- enantiomers are co-eluted</td>
</tr>
<tr>
<td>4.</td>
<td>Column: chiralpak AD-H 250 x 4.6 mm, 5 µm, Mobile phase: n-hexane, IPA and TEA (80:20:0.1, v/v/v), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 6.0 R-enantiomer:6.0</td>
<td>S and R- enantiomers are co-eluted</td>
</tr>
<tr>
<td>5.</td>
<td>Column: chiralcel OD-H 250 x 4.6 mm, 5 µm, Mobile phase: n-hexane, IPA and TEA (80:20:0.1, v/v/v), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 4.8 R-enantiomer:4.8</td>
<td>S and R- enantiomers are co-eluted</td>
</tr>
<tr>
<td>6.</td>
<td>Column: crownpak CR (+) 150 x 4.6 mm, 5 µm, Mobile phase: 0.1 M HClO(_4), Flow rate: 1.0 mL min(^{-1})</td>
<td>S-enantiomer: 2.5 R-enantiomer: 2.9</td>
<td>Resolution is poor between enantiomers.</td>
</tr>
<tr>
<td>7.</td>
<td>Column: chiral AGP 150 x 4.6 mm, 5 µm, Mobile phase: 0.01 M KH(_2)PO(_4):Methanol (50:50, v/v), pH:4.0, Flow rate: 1.0 mL min(^{-1}), Column temperature: 25 °C</td>
<td>S-enantiomer: 6.2 R-enantiomer: 6.2</td>
<td>S and R- enantiomers are co-eluted.</td>
</tr>
<tr>
<td>8.</td>
<td>Column: chirobiotic T 150 x 4.6 mm, 5 µm, Mobile phase: 0.02 M KH(_2)PO(_4): Methanol (90:10, v/v), pH : 4.0, Flow rate: 1 mL min(^{-1}), Column temperature: 25 °C</td>
<td>S-enantiomer: 5.5 R-enantiomer: 5.5</td>
<td>S and R- enantiomers are co-eluted.</td>
</tr>
</tbody>
</table>

(b) System suitability test results

<table>
<thead>
<tr>
<th>Name</th>
<th>Retention time (Rt) in min</th>
<th>Resolution (Rs) by tangent method (USP)</th>
<th>USP tailing factor (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-enantiomer</td>
<td>7.7</td>
<td>--</td>
<td>1.05</td>
</tr>
<tr>
<td>R-enantiomer</td>
<td>8.4</td>
<td>2.0</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Solution Stability and Mobile Phase Stability

Solution stability was studied by keeping the test solution in tightly capped volumetric flasks at room temperature on a laboratory bench for 24 h. The content of (R)-enantiomer was checked for every 6 h interval and compared with freshly prepared solution. No variation was observed in the
content of the (R)-enantiomer for the study period and it indicates (S)-2-azido-3-methylbutanoic acid sample solutions prepared in diluent were stable up to 24 h. Mobile phase stability was carried out by evaluating the content of (R)-enantiomer in (S)-2-azido-3-methylbutanoic acid sample solutions, which were prepared freshly at every 6 h interval for 24 h. The same mobile phase was used during the study period. No variation was observed in the content of (R)-enantiomer for the study period and it indicated the prepared mobile phase was stable up to 24 h.

Fig. 1. Chemical structure of (S) and (R)-2-azido-3-methylbutanoic acid

(S)-2-azido-3-methylbutanoic acid

(R)-2-azido-3-methylbutanoic acid

Fig. 2. LC chromatogram of S-enantiomer spiked with R-enantiomer
CONCLUSION

A simple and accurate normal phase chiral LC method was developed for the quantitative determination of the (R)-enantiomer in (S)-2-azido-3-methylbutanoic acid, a key starting material of valganciclovir hydrochloride. Chiralpak IA, an amylose based chiral stationary phase was found to be selective for the enantiomers of 2-azido-3-methylbutanoic acid. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method can be used for the quantitative determination of the undesired (R)-enantiomer in 2-azido-3-methylbutanoic acid samples.

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