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**Research Article**

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# Separation of the two enantiomers of Gatifloxacin by SFC on amylose based stationary phase

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**ABSTRACT**

*A Supercritical fluid liquid chromatographic method has been developed in normal-phase conditions for the separation of enantiomeric Gatifloxacin, (±) 1-cyclopropyl-6-fluoro-8-methoxy-7- (3-methylpiperazin-1-yl)-4-oxo-quinoline-3-carboxylic acid, an antibiotic in bulk drug. The method involved the use of an amylose based Chiralpak AD-H (250 mm x 4.6 mm, 5 μm) column using a mobile phase system containing Supercritical fluid ($Co_2$),methanol and diethylamine (90:10:0.5% v/v). The conditions affording the best resolution were found by selection and variation of the mobile-phase compositions and the differences in separation capability of the method is noted. Relative standard deviation of retention times and peak areas were better than 0.2% and 0.4%, respectively, for precision. Gatifloxacin sample solution and mobile phase are found to be stable for at least 48 h.*

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## INTRODUCTION

The fluoroquinolones have emerged as one of the most important class of antibiotics, and many of these compounds are chiral in nature. Chirality to these compounds is either imparted by presence of stereogenic center in the side chain part (e.g., gatifloxacin, moxifloxacin, lomefloxacin, sparfloxacin, clinafloxacin, etc.) or core part (e.g., flumequin, ofloxacin, WCK 771) [1]. Gatifloxacin ((±)-1-cyclopropyl-6-fluoro- 8-methoxy-7-(3-methylpiperazin- 1-yl)- 4-oxo-quinoline-3-carboxylic acid) (Figure 1) is a broad-spectrum 8-methoxy fluoroquinolone and is used for the treatment of infections of the respiratory and genitourinary tracts [2]. Severalmethods have reported for enantiomeric separation of these fluoroquinolone compounds [1]. These methods include derivatization to diastereomers [3–6], chiral mobile phase-based on ligand exchange [7–9], and chiral stationary phase (CSP) methods. Predominantly use of protein based CSPs and crown ether-based CSPs have been reported [10–14].

In the recent years, there has been increasing concern over drug stereochemistry as the result of the increasing realization of the significance of the pharmacodynamic and pharmacokinetic differences between the enantiomers of chiral drugs [15]. Several means for determining the enantiomeric content of chiral fluoroquinolones have been reported. High-performance liquid chromatograhy (HPLC) as powerful analytical tool has been widely used for determining the enantiomers of chiral fluoroquinolones. The HPLC separation of fluoroquinolone enantiomers require the use of various adaptations to be successful, such as the use of a chiral stationary phase (CSP), the addition of chiral selectors to themobile phase, or derivatization of the analyte with a suitable chiral reagent, due to

differences in their chemical structure and in the location of chiral center(s). The carboxyl group, which is common to all fluoroquinolones, allows the separation of the enantiomers of different fluoroquinolones by ligand exchange method or after derivatization. But this approach fails to separate the stereoisomers of gatifloxacin due to the remoteness of the chiral center at the 7-substitutent from the carboxylic group (Figure 1).

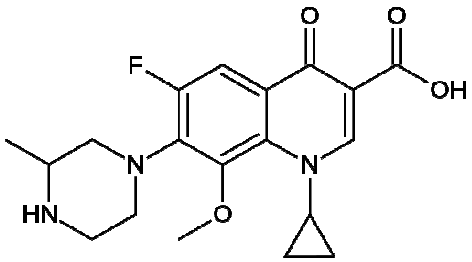



**Figure.1 Chemical structure of Gatifloxacin.**

The liquid chromatographic resolution of enantiomers on CSP is known as one of themost convenient and accuratemeans of determining the enantiomeric composition of chiral compounds. For the enantiomeric separation of quinolones, results have been reported only for crown ether, amylose, cellulose, or protein-based columns (1). Crown ether-based CSPs are efficient in resolving the enantiomers of several quinolones, but their use is restricted to separation of the stereoisomers of quinolone derivatives that bear an amino or aminomethyl substituent in close proximity to a chiral center.

The objective of the present investigation is to develop a simple, reliable, and direct normal-phase HPLC method for the enantiomeric separation of gatifloxacin using an amylose-based CSP column. The mobile phase in normal phase mode is simple to prepare and the method is rugged in any environment and comfort in use.

## EXPERIMENTAL SECTION

### Chemicals and reagents

Gatifloxacin racemic mixture (±) was obtained from the R&D department of Dr.Reddys laboratories (Hyderabad, India). Chemical structure is presented in Figure 1. HPLC-grade methanol was purchased from Merck (Mumbai, India), and HPLC-grade diethyl amine was purchased from Spectrochem (Mumbai, India). All other chemicals were of analytical-grade, Carbon dioxide with high purity was supplied from MRG Enterprises Hyderabad, India.

The analyte concentration of Gatifloxacin was fixed as 1mg/mL. Working solutions of Gatifloxacin were prepared in Methanol (50:50, v/v).

### Apparatus

The SFC experiments were performed on a Thar SD-ASFC-2 system (Thar Technologies, Pittsburgh, PA) equipped with a Waters PDA detector. The system was controlled by Superchrom software. The enantioseparation was performed on a Chiralpak AD-H [Amylose tris (3,5-dimethylphenylcarbamate) coated on 5µm silica-gel), 250 X 4.6 mm i.d., 5 µm]. The chromatography was conducted using CO2 composed with different types and percentages of alcohol modifiers as the mobile phase. For the separation experiments, the total flow rate of the mobile phase, the outlet backpressure, the injection volume, and the UV detection wavelength were fixed at 2.5 mL/min, 100 bars, 20 µL, and 225 nm, respectively. The oven temperature was set at 25°C, unless noted otherwise, to determine the effect of temperature on enantiomeric separation. The chromatographic parameters, including the retention factor (k), the separation factor (α), and the resolution (Rs) were selected to evaluate the separation of the compounds. All the chromatographic results were repeated three times.

Different chiral columns were also employed during method development namely Chiralcel OD-H (250 × 4.6 mm,5µ), Chiralpak IC (250 × 4.6 mm,5µ),Amylose-2(250 × 4.6 mm,5µ), Chiralcel OJ-H (250 × 4.6 mm,5µ), Chiralpak AS-H (250 × 4.6 mm,5µ), Chiralpak AD-H (250 × 4.6 mm,5µ).

**Validation of the method**

**Precision**

Precision was determined by measuring repeatability and reproducibility of retention times and peak areas of each enantiomer. In order to determine the repeatability of the method, replicate injections (n = 6) of standard was carried out from single preparation. The reproducibility of the method was determined by analyzing three different preparations of test solutions containing gatifloxacin (0.8 mg/mL).

**Linearity**

Linear response was determined by preparing and analyzing five calibration solutions of the enantiomers (concentration from 0.4 mg/mL to 1.2 mg/mL) in methanol. 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 were calculated.

**Robustness**

The robustness of a method is the ability of the method to remain unaffected by small changes in parameters such as flow rate, mobile phase composition, and column temperature. To determine robustness of the method experimental conditions were purposely altered and chromatographic resolution between (+) enantiomer and (–) enantiomer was evaluated. The flow rate of the mobile phase was 2.50 mL/min. To study the effect of flow rate on the resolution of enantiomers, 0.5 units (±10%) changed it from 2.0 to 3.0 mL/min. The effects of change in percent of Supercritical fluid Carbon dioxide, and methanol on resolution were studied by varying from –2 to +2% while the other mobile phase components were held constant as stated in the Apparatus section.

## RESULTS AND DISCUSSION

**Optimization of chromatographic conditions**

Racemic mixture solution of gatifloxacin (10 µg/mL each) prepared in methanol for development. To develop a rugged and suitable SFC method for the separation of the two enantiomers, different stationary phases and mobile phases were employed. Initial screening of chiral column was carried out by several chiral LC column suppliers. Various chiral columns, namely Chiralcel OD-H (250 × 4.6 mm,5µ), Chiralpak IC (250 × 4.6 mm,5µ),Amylose-2(250 × 4.6 mm,5µ), Chiralcel OJ-H (250 × 4.6 mm,5µ),Chiralpak AS-H (250 × 4.6 mm,5µ), Chiralpak AD-H (250 × 4.6 mm,5µ).were employed. All these columns failed to provide selectivity between gatifloxacin peak and the undesired enantiomer peak using different possible mobile phases. Different method development trails are shown in (Fig 2). Different method development trail conditions are given in Table.1.

It was continued to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. There was an indication of separation on Chiralpak AD-H (250 × 4.6 mm, 5 µm) column using a mobile phase consisting of Supercritical fluid liquid ($Co_2$), methanol and diethyl amine (90:10:0.5%,v/v). The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. The results of resolution factor (Rs) and selectivity factor (α) are getting good results. Based on the data obtained from method development and optimization activities, Chiralpak AD-H (250 × 4.6 mm, 5 µm) column with mobile phase of Supercritical fluid liquid ($Co_2$), methanol and diethyl amine (90:10:0.5%,v/v) was selected for the final method. The flow rate of the final method was 2.5 mL/min with an injection volume of 20 µL. The column temperature was 25°C, and the detection wavelength was 225 nm. Under these conditions, the two enantiomers were separated well and the peak of (*R*)-enantiomer eluted after the peak of Gatifloxacin. In the optimized method, the typical retention times of Gatifloxacin and (*R*)-enantiomer were approximately 4.72 and 6.58 min, respectively. Baseline separation of Gatifloxacin and (*R*) - enantiomer was obtained with a total run time of 10 min. The separation of an approximately 1:1 (wt/wt) mixture solution (in methanol) of the two enantiomers is shown in Figure 2. An amylose based chiral stationary phase contained five chiral centers per unit, and Gatifloxacin had only one chiral center close to the methyl group in the structure. The stereoelectronic interactions between the enantiomers and the chiral stationary phase generated enantioselectivity, thus causing significant differences in the migration of the enantiomers inside the column. Having the right amount of methanol in the mobile phase also played an important role in affecting the steric environment of the chiral cavities or channels of the stationary phase and contribute to enantioselectivity. However, an excessive amount of methanol was likely to cut down the resolution by taking up chiral centers of the chiral stationary phase or forming hydrogen bondings with the enantiomers instead of the hydrogen bondings between the enantiomers and the stationary phase (14). Other

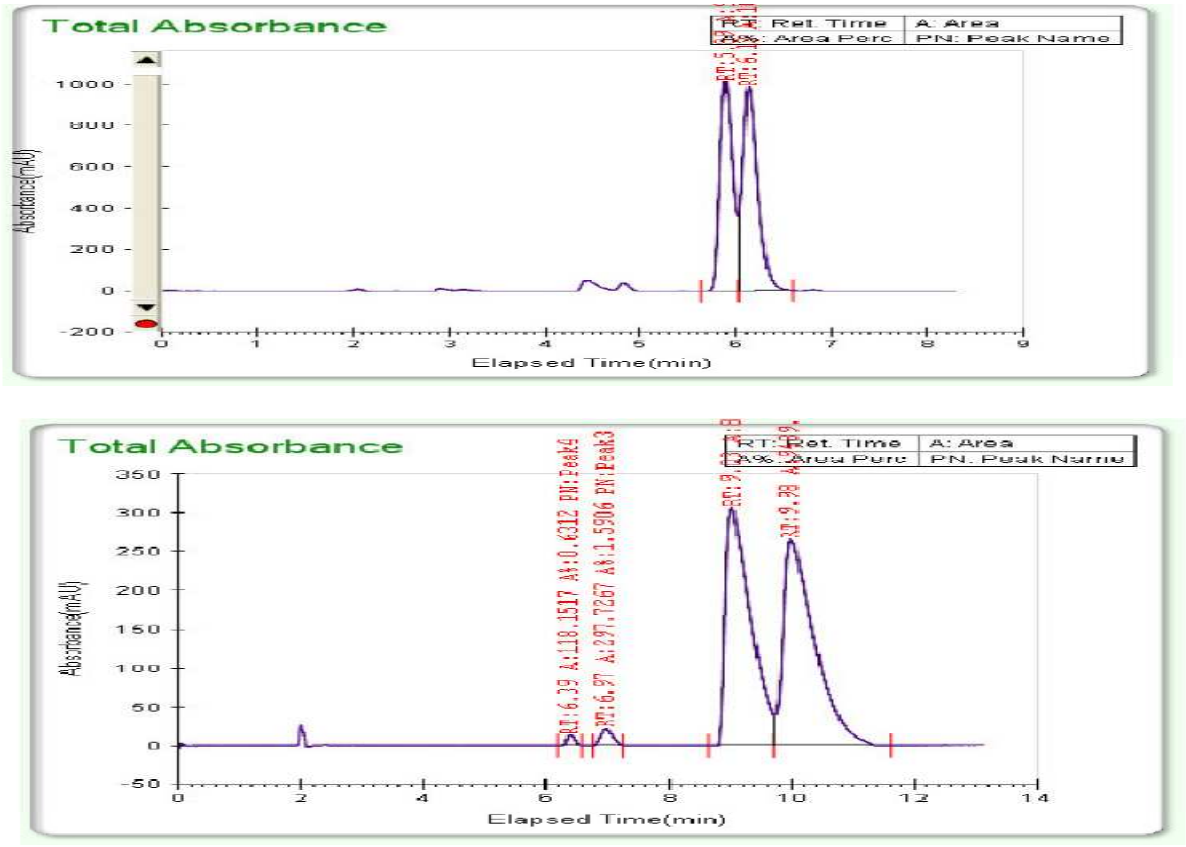
important interactions between the enantiomers and the stationary phase such as π–π bonding, Vander Waals forces, dipole induced dipole attractions, and steric effects can also achieve better resolution on Chiralpak AD-H column.

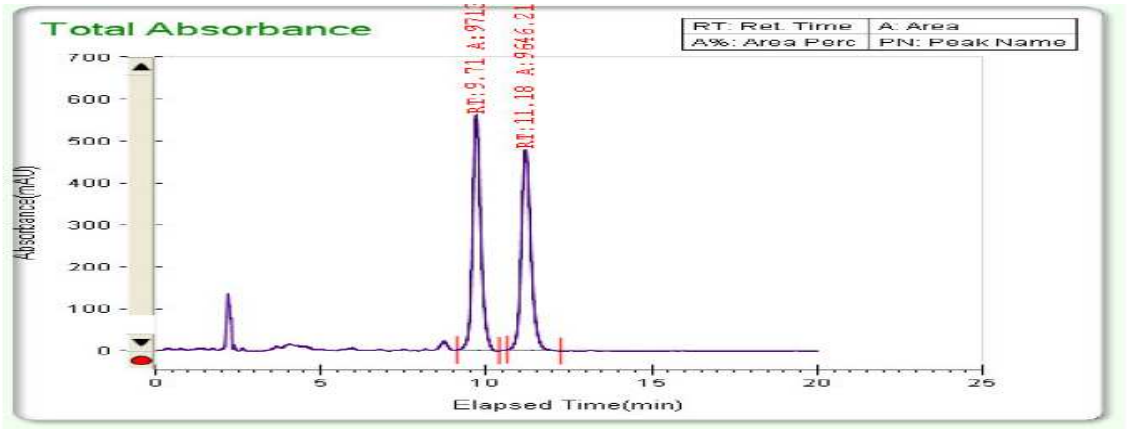
**Validation of method**

In the repeatability study, the relative standard deviation (RSD) was better than 0.2% for the retention times and 0.4% for peak areas of both the enantiomers. In reproducibility studies, results show that RSD values were in the same order of magnitude than those obtained for repeatability. Coefficient of correlation between concentration and detector response [0.999 for the first eluting (+) enantiomer and 0.999 for the second eluting (–) enantiomer] shows that method is linear over the concentration range of 0.5–1.5 mg/mL .The limit of detection (LOD), determined as the amount for which the signal to- noise ratio was ~3:1 for both enantiomers of gatifloxacin, was 0.05 mg/mL. SFC chromatogram of (±)-enantiomer of Gatifloxacin sample was shown in (fig.2)

The chromatographic resolution of Gatifloxacin peaks were used to evaluate the method robustness under modified conditions. The resolution between (+) enantiomer and (-) enantiomer was greater than 2.0 under all separation conditions tested demonstrating sufficient robustness.

**Figure II. Different method development trails.**





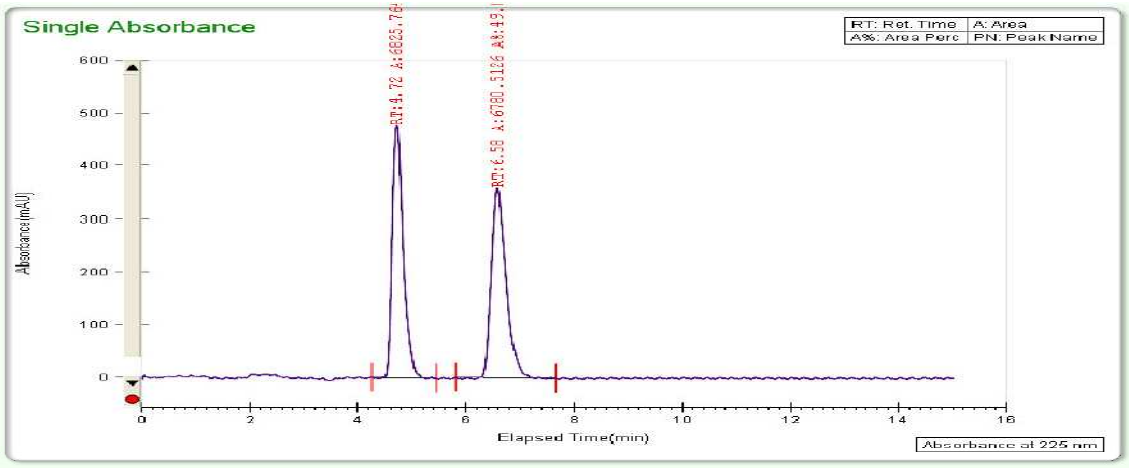




**Table I. Different method development trail conditions**

| S.No | Column name | Total flow(g/min) | % of Cosolvent | Remarks |
|---|---|---|---|---|
| 1 | Chiralcel OD-H | 2 | 15% Methanol | Poor separation |
| 2 | Amylose -2 | 2 | 15%(IPA) | Poor resolution |
| 3 | Chiralcel OJ-H | 2 | 20%(0.5% TFA in ACN) | Poor resolution |
| 4 | ChiralPak AD-H | 2.5 | 10%(0.5% DEA in MeoH) | Good Separation |

## CONCLUSION

A simple, rapid, and accurate Supercritical fluid liquid chromatographic method for the enantiomeric separation of Gatifloxacin has been developed and validated. Amylose based Chiralpak AD-H column was found to be suitable for the separation of the enantiomers of Gatifloxacin. Use of Chiralpak AD-H column with Supercritical fluid liquid ($CO_2$), methanol and diethylamine (90:10:0.5%, v/v) as mobile phase was most suitable for separation of the enantiomers of Gatifloxacin. The analysis of enantiomeric separations by SFC is less time, cost effective and environmentally eco friendly than Normal phase liquid chromatographic Methods.

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