# High-Performance Liquid Chromatographic Method For The Separation of Enantiomeric Gatifloxacin

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## Abstract

**A high-performance 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 (150 mm x 4.6 mm, 5 µm) column using a mobile phase system containing n-hexane–ethanol–diethylamine (85:15:0.1% 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.**

## 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). Several methods 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 the mobile 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).

The liquid chromatographic resolution of enantiomers on CSP is known as one of the most convenient and accurate means 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.

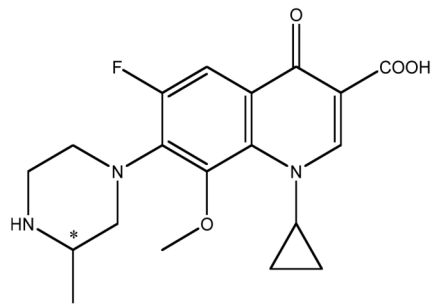


**Figure 1.** Chemical structure of gatifloxacin (±) enantiomer.

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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

### Chemicals and reagents

Gatifloxacin racemic mixture (±) was obtained from the R&D department of Suven Life Sciences Limited (Hyderabad, India). Chemical structure is presented in Figure 1. HPLC-grade *n*-hexane was purchased from Merck (Mumbai, India). HPLC-grade ethanol was purchased from Hayman Limited (Essex, England), and HPLC-grade diethylamine was purchased from Spectrochem (Mumbai, India). HPLC water from Milli-Q system (Millipore, Bedford, MA) was used. Aqueous buffers $KPF_6$, $CH_3COONa$ and $Na_2HPO_4$ were purchased from Merck (Worli, Mumbai). All other chemicals were of analytical-grade.

The analyte concentration of gatifloxacin was fixed as 1 mg/mL. Working solutions of gatifloxacin were prepared in a mixture of mobile phase and ethanol (50:50, v/v).

### Apparatus

Chiral separations were carried on 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a G1311A quaternary pump, a G1313A degasser, a G1313A autosampler, a G1316A thermostatted column compartment, and a G1314A UV-detector. Data was processed using computer program Chemstation.

The chromatographic conditions were optimized using a chiral stationary phase Chiralpak AD-H (150 × 4.6 mm, 5 µm, Daicel, Tokyo, Japan), which was safeguarded with a 1 cm length guard column. The isocratic mobile phase composition was a mixture of containing *n*-hexane–ethanol–diethyl amine (85:15:0.1%, v/v), which was pumped at a flow rate of 0.5 mL/min. The column was maintained at 30°C temperature, and the eluent was monitored at a wavelength of 220 nm. The injection volume was 10 µL. Different chiral columns were also employed during method development namely Chiralcel OD-RH (150 × 4.6 mm), Chiralcel OJ-RH (150 × 4.6 mm), Chiral AD-RH (150 × 4.6 mm), Chiral AS-H (150 × 4.6 mm), Chiral OD-H (150 × 4.6 mm) and Chiralpak AD-H (150 × 4.6 mm), all produced by Daicel, Chiral AGP (150 × 4.0 mm), Chiral HSA (150 × 4.0 mm), last two produced by Chromtech.

### 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 (1 mg/mL).

*Linearity*

Linear response was determined by preparing and analyzing five calibration solutions of the enantiomers (concentration from 0.5 mg/mL to 1.5 mg/mL) in (1:1) mobile phase and ethanol. 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 0.50 mL/min. To study the effect of flow rate on the resolution of enantiomers, 0.05 units (±10%) changed it from 0.45 to 0.55 mL/min. The effect of change in percent of *n*-hexane and diethylamine on resolution was studied by varying from –2 to +2% while the other mobile phase components were held constant as stated in the Apparatus section.

### Selection of mobile phase

Different mobile phases were tested for their suitability for the separation of the enantiomers on the chiralpak AD-H (150 mm × 4.6 mm) column. For each determination, capacity, selectivity, and resolution factors were measured.

$$k_1' = \frac{[\text{RT of the first eluted enantiomer} - \text{dead time}]}{\text{dead time}}$$

$$k_2' = \frac{[\text{RT of the second eluted enantiomer} - \text{dead time}]}{\text{dead time}}$$

$$\alpha = k_2'/k_1'$$

$$R_S = 2\times \frac{[\text{difference of retention time of (+) and (–) enantiomers}]}{[\text{the bandwidths of the two peaks}]}$$

where $k_1'$ is capacity factor of the first eluted enantiomer; $k_2'$ capacity factor of the second eluted enantiomer; $\alpha$ is the selectivity factor; $R_S$ is resolution factor; and RT is the retention time.

## Results and Discussion

### Selection of suitable reverse-phase column

The aim of this work is to separate the enantiomers of gatifloxacin and accurate quantification of individual enantiomer. The stock solution of 1 mg/mL was prepared in mobile phase and ethanol (50:50) used in the method development. To develop a rugged and suitable LC method for the separation of gatifloxacin

enantiomers, different mobile phases and stationary phases were employed. The main target of the chromatographic method is to separate the racemic mixture of gatifloxacin into individual enantiomers using simple mobile phase. Various reverse-phase chiral columns namely Chiralcel OD-RH, Chiralcel OJ-RH, Chiral AD-RH of Daicel and Chiral AGP, Chiral HSA of Chromtech and normal-phase chiral columns namely Chiralcel OJ-H, AS-H, and OD-H were employed. But unfortunately, no proper separation (resolution greater than 2.0) was found on these columns using different possible mobile phases of 25 mM $KPF_6$–acetonitrile; 25 mM $KPF_6$–methanol; 25 mM $CH_3COONa$–acetonitrile; 25 mM $Na_2HPO_4$–acetonitrile; *n*-hexane–isopropanol. The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. Best separation was achieved on Chiralpak AD-H column using the mobile phase composition of *n*-hexane–ethanol–diethylamine (85:15:0.1% v/v). Due to better chromatographic results obtained on the Chiralpak AD-H column, the method validation was carried out using this column.

**Table I. Parameters Determined for Separation of the Gatifloxacin Enantiomers***

| Mobile phase | $k_1'$ | $k_2'$ | α | RS |
|---|---|---|---|---|
| *n*-Hexane–ethanol–diethylamine (85/15/0.1%) | 2.36 | 3.08 | 1.31 | 2.51 |
| *n*-Hexane–ethanol–diethylamine (85/15/0.08%) | 4.10 | 5.10 | 1.24 | 2.02 |
| *n*-Hexane–ethanol–diethylamine (85/15/0.12%) | 3.50 | 4.33 | 1.24 | 2.46 |
| *n*-Hexane–ethanol–diethylamine (87/13/0.1%) | 5.47 | 7.00 | 1.28 | 2.58 |
| *n*-Hexane–ethanol–diethylamine (83/17/0.1%) | 3.42 | 4.39 | 1.29 | 3.39 |
| *n*-Hexane–ethanol–diethylamine (85/15/0.1%) 0.55 mL/min | 4.45 | 5.69 | 1.28 | 2.81 |
| *n*-Hexane–ethanol/Diethylamine (85/15/0.1%) 0.45 mL/min | 4.49 | 5.73 | 1..28 | 2.75 |

* On column Chiralpak AD-H (150 mm x 4.6 mm) column, flow rate 0.5 mL/min, 30°C.

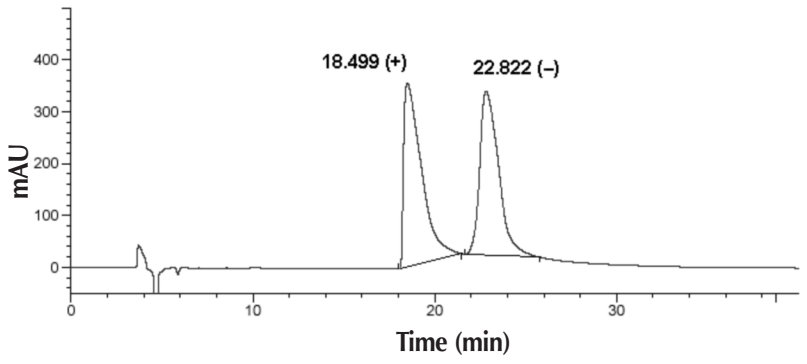


**Figure 2.** Enantiomeric resolution of Gatifloxacin on Chiralpak AD-H column.

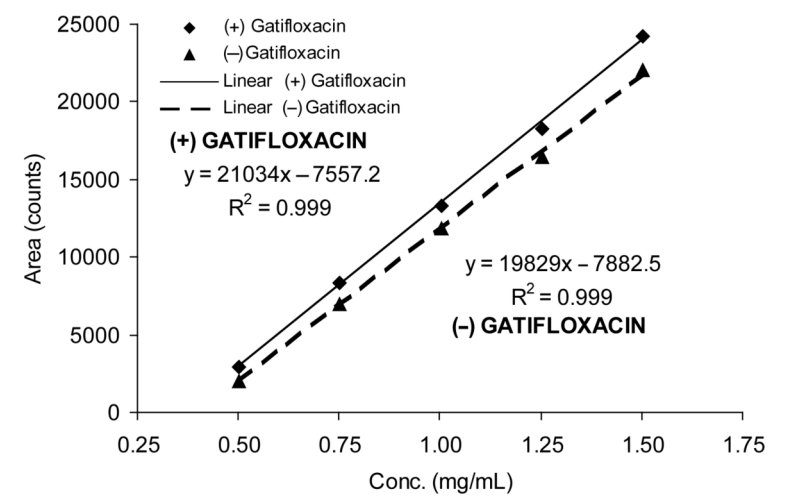


**Figure 3.** Linearity curve of (A) Gatifloxacin (+) enantiomer and (B) Gatifloxacin (–) enantiomer on Chiralpak AD-H column.

### Optimization of chromatographic conditions

Initially, the mobile phase consisting of *n*-hexane and isopropanol was used to separate the racemic mixture. But broadening of peaks was observed, and the retention time was longer. Then, some amount of (0.05 %) diethylamine was introduced to reduce the peak broadening. In this case the two enantiomers were started merging, and separation was disturbed. Finally, isopropanol was replaced with ethanol and the mobile phase consisting of *n*-hexane, ethanol and diethylamine in the ratio of 85:15:0.1% (v/v). In this mobile phase good peak shape and good resolution, which is greater than 2, was achieved.

In the optimized method, the typical retention times of (+) and (-) enantiomer of gatifloxacin were about 18.5 and 22.8 min, respectively. The enantiomeric separation of gatifloxacin on Chiralpak AD-H column was shown in Figure 2. Baseline separation of (+) enantiomer ($k_1'$=2.36) and (–) enantiomer ($k_2'$ =3.08) was obtained with a total run time of 40 min.

### 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 (Figure 3). 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. A HPLC chromatogram of (±)-enantiomer of gatifloxacin sample was shown in Figure 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 (Table I), demonstrating sufficient robustness.

## Conclusions

A simple, rapid, and accurate normal-phase chiral LC 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 *n*-hexane–ethanol–diethylamine (85:15:0.1%, v/v) as mobile phase was most suitable for separation of the enantiomers of gatifloxacin.

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