Development and Validation of an Ion-Pairing RP-HPLC Method for the Estimation of Gatifloxacin in Bulk and Formulations

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Abstract

A new, simple, and sensitive ion-pair reverse-phase liquid chromatographic method is developed and validated for the estimation of 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (gatifloxacin) in bulk and formulations using a UV detector under isocratic conditions. The selected mobile phase consists of the aqueous phase (a 25mM citrate buffer comprising of 10mM cationic and anionic pairing agents, pH adjusted to 3.5) and acetonitrile (52:48%, v/v). The selected wavelength is 292 nm. Retention time of gatifloxacin is 5.2 min. The linearity range found is 50 to 1000 ng/mL (the regression equation is area = 105.5 × concentration in ng/mL – 695.8), and the regression coefficient is 0.9996. Validation results demonstrate accuracy, precision, and reproducibility (relative standard deviation < 3%) of the method. The detection and quantitation limits are found to be 6.50 and 17.38 ng/mL, respectively. The method is successfully used for the estimation of gatifloxacin in a variety of dosage forms, and the results are in good agreement with the label claims.

Introduction

Gatifloxacin is a fourth-generation fluoroquinolone derivative [1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid] with a broad spectrum of activity encompassing gram-positive and gram-negative pathogens, including S. epidermidis, S. aureus, S. pneumoniae, S. pyogenes, H. influenzae, E. coli, B. cereus, N. gonorrhoeae, and P. mirabilis. Additionally, gatifloxacin is highly active against atypical pathogens, such as Mycoplasma, Legionella, and Chlamydia species, as well as the anaerobic organism P. acnes (1–3). Because of its clinical advantages, there is an increase in the number of gatifloxacin formulations in the market for a variety of indications. Therefore, there is a need for a sensitive and reliable analytical method for the estimation of gatifloxacin in pharmaceutical formulations. A single method that can be used for the estimation of a variety of formulations will give an additional advantage.

Microbiological assay and high-performance liquid chromatography (HPLC) methods (with electrospray tandem mass spectrometry, UV and fluorescence detectors) were reported for the estimation of gatifloxacin in biological fluids, such as plasma, serum, and urine (4–9). Apart from UV-spectrophotometry (10,11) and microbiological assay (12) methods, two HPLC methods (13,14) have been reported in the literature. Because of the low sensitivity of gatifloxacin with a reported limit of quantitation (LOQ) of 390 (13) and 200 ng/mL (14), these HPLC methods cannot be applied to ophthalmic formulations. Thus, there is a need to develop a sensitive, accurate, and precise method with improved gatifloxacin peak properties.

The present study was aimed at developing a sensitive ion-pair reverse-phase (RP)-HPLC method suitable for routine and selective analysis of gatifloxacin in a variety of commercially available pharmaceutical preparations and samples of very low concentration. A mobile phase was used for the extraction of the drug from the formulations in order to reduce the sample preparation steps. The HPLC separation was achieved in isocratic RP (C-18) conditions. The use of ion-pairing agents improved the drug peak properties. The LOQ and limit of detection (LOD) were also found to be lower than the reported methods. The developed method was validated as per ICH guidelines (15) and the United States Pharmacopoeia requirements (16). The proposed method was successfully used for the estimation of gatifloxacin in tablet, injection, and ophthalmic preparations. The developed method can be used to estimate gatifloxacin at a very low level.

Experimental

Materials and apparatus

Gatifloxacin was obtained as a gift sample from Venkar Chemicals Pvt. (Hyderabad, India). Tetrabutyl ammonium
hydrogen sulphate (TBAHS) and sodium dodecyl sulphate (SDS) were purchased from LOBA Chemi (Mumbai, India). Triethyl amine (TEA) and acetonitrile (ACN) were of HPLC grade and purchased from Spectrochem (Hyderabad, India). All other buffer salts were of analytical grade. Triple distilled water was prepared in-house and used for the buffer preparation. The aqueous phase was filtered through 0.22 µm filters (Millipore, Bedford, MA) after preparation. The excipients used (lactose, starch, methyl cellulose, polyethylene oxide, eudragit, hydroxypropylmethylcellulose, microcrystalline-cellulose, dextrose, iron oxide yellow, titanium oxide, magnesium stearate, talc, and benzalkonium chloride) were either purchased from a local market or provided by IPCA Laboratories (Mumbai, India) and Ranbaxy Research Laboratories (Mumbai, India).

Formulations containing gatifloxacin were as follows: Gaity-200 tablets, labeled to contain 200 mg of gatifloxacin per tablet (Dr. Reddy's Laboratories, Gurgaon, India), Gaity-400 tablets, labeled to contain 400 mg of gatifloxacin per tablet (Dr. Reddy's Laboratories); Gatilox concentrated injection, labeled to contain gatifloxacin 10 mg/mL (Solares, Sun Pharma, India); and Zymar eye drops, labeled to contain gatifloxacin 0.3% (w/v) (Nicholas Piramal India, Mumbai, India). They were collected from a local Indian market. Gatifloxacin ophthalmic inserts of 150-µg strength were prepared in the laboratory using hydroxypropyl methylcellulose as the polymeric carrier under aseptic conditions. Apart from common excipients, Gaity-200 and Gaity-400 tablets contained excipients like iron oxide yellow and titanium oxide. The Gatilox concentrated injection contained excipients like dextrose and water for injection. Gatifloxacin eye drops contained 0.01% (w/v) of benzalkonium chloride (preservative) in an aqueous vehicle.

HPLC equipment (Jasco, Tokyo, Japan) consisted of model PU-1580 intelligent HPLC pumps, a UV-1575 model intelligent UV–vis detector, and an AS-1559 model intelligent autosampler. Separation was performed on a Hibar LiChroSpher 100 RP-18e (250 × 4-mm, 5 µm particle size) column under isocratic conditions. Chromatograms were analyzed using the Borwin software provided with the system. The pH of the solutions was adjusted with an Elico pH meter (Elico, Hyderabad, India).

**Chromatographic condition**

The mobile phase consisted of an aqueous phase (25 mM citrate buffer, 10 mM TBAHS, and 10 mM SDS in triple distilled water, pH adjusted to 3.5) and ACN (52:48%, v/v). Before use, the mobile phase was filtered through a 0.22 µm filter and degassed by sonication. The injection volume was 20-µL. The flow rate was adjusted to 1 mL/min, the sensitivity was 0.0005 AUFS, and the wavelength of the UV detector was set at 292 nm. All the experiments were conducted at room temperature at approximately 25°C. The system was stabilized for at least 1 h before analysis.

**Mobile phase composition optimization**

Different pH media (pH 3 to 5 orthophosphoric acid–triethyl amine buffer, pH 3 to 5 ammonium acetate buffer, pH 3 to 5 phosphate buffer, and pH 3 to 5 citrate buffer), in combination with different organic solvents (ACN and methanol) and ion pairing agents in various proportions (TEA, SDS, and TBAHS), were tried. For the selection of media, the criteria employed was gatifloxacin peak properties (retention time and asymmetric factor), sensitivity (height and area), ease of sample preparation, and applicability of the method for various purposes.

**Calibration curve**

A primary stock solution of 50 µg/mL of gatifloxacin was prepared in the mobile phase. From an aliquot of primary stock, secondary stock solution of 1 µg/mL was prepared in the mobile phase. For preparation of different concentrations, aliquots of primary or secondary stock solutions were transferred into a series of 5-mL standard flasks and volume was made with the mobile phase. Six different concentrations (50, 100, 250, 500, 750, and 1000 ng/mL) of gatifloxacin were prepared for the calibration curve. Twenty microliters of each concentration was injected, and the area of the peak was determined (Table I). To establish linearity of the proposed method, six separate series of solutions of the drug in a selected medium were prepared from the stock solution and analyzed. The average area of each concentration was substituted in a regression equation to calculate the corresponding predicted concentration. A least square regression analysis was performed for the obtained data. An analysis of variance test (one-way) was performed based on the peak area observed for each pure drug concentration during the replicate measurement of the standard solutions (17).

**Analytical validation**

To study selectivity of the method, gatifloxacin solutions (50 µg/mL) were separately prepared in a mobile phase with and without common excipients (lactose, starch, methyl cellulose, polyethylene oxide, eudragit, hydroxypropylmethylcellulose, microcrystalline cellulose, dextrose, iron oxide yellow, titanium oxide, magnesium stearate, talc, and benzalkonium chloride). All the solutions were diluted suitably with the mobile phase to get a drug concentration of 1000 ng/mL, and they were analyzed. A blank solution containing only excipient was also injected after similar dilutions were made, and interference near the drug peak was checked.

As a part of determining the accuracy of the proposed method, different levels of drug concentrations [lower quality control samples (LQC) = 75 ng/mL, medium quality control

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Mean area (µV-sec) (± SD)</th>
<th>% RSD</th>
<th>Predicted conc. (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5024.9 ± 92.6</td>
<td>1.84</td>
<td>52.2</td>
</tr>
<tr>
<td>100</td>
<td>10090.9 ± 115.9</td>
<td>1.15</td>
<td>102.2</td>
</tr>
<tr>
<td>250</td>
<td>25262.6 ± 276.9</td>
<td>1.10</td>
<td>246.0</td>
</tr>
<tr>
<td>500</td>
<td>51533.0 ± 1011.9</td>
<td>1.96</td>
<td>495.0</td>
</tr>
<tr>
<td>750</td>
<td>78224.5 ± 624.4</td>
<td>0.80</td>
<td>747.9</td>
</tr>
<tr>
<td>1000</td>
<td>105306.9 ± 658.7</td>
<td>0.63</td>
<td>1004.6</td>
</tr>
</tbody>
</table>
samples (MQC) = 400 ng/mL, and higher quality control samples (HQC) = 900 ng/mL were prepared independently from stock solution and analyzed (n = 6). Accuracy was assessed as the percentage relative error and mean percentage recovery. Further, different concentrations of pure drug (50, 100, 250, and 500 ng/mL) were added to a known pre-analyzed formulation sample (drug concentration of 507.9 ng/mL) and analyzed using the proposed method (n = 5) to check the accuracy. The percent recovery of the added pure drug was calculated as, % Recovery = [(Cv – Cu)/Ca] × 100, where Cv is the total drug concentration measured after standard addition, Cu is the drug concentration in the formulation, and Ca is the drug concentration added to formulation.

Repeatability was determined by using different levels of drug concentrations (as mentioned in accuracy), prepared from independent stock solution and analyzed (n = 6) (Table II). Inter- and intra-day variation and analyst variation was studied to determine intermediate precision of the proposed method. Different levels of drug concentrations in triplicates were prepared twice in a day and studied for intra-day variation. The same protocol was followed for three different days to study inter-day variation (n = 18). Different analysts prepared different solutions on different days. The relative standard deviation (RSD) (in %) of the predicted concentrations from the regression equation was taken as precision.

The LOD and LOQ of gatifloxacin by the proposed method was determined using a signal-to-noise (S/N) ratio. An S/N ratio of 3 and 10 were taken as LOD and LOQ, respectively (15). The LOQ samples were prepared in replicates (n = 5) using the same procedure followed for calibration standards and were analyzed. Robustness of the proposed method was determined by varying the pH of the media by ± 1 units and using bench-top stability and stock solution stability of gatifloxacin at room temperature for 36 h.

Estimation from formulations

Twenty tablets were weighed and pulverized (average tablet weight of 316.86 mg for Gaity-200 and average tablet weight of 651.88 mg for Gaity-400). An amount of the powder equivalent to 5 mg of gatifloxacin was accurately taken and sonicated with the mobile phase for 5 min. The solution was diluted suitably to prepare a 50 µg/mL concentration. This primary stock solution was filtered through Whatman filter paper number 40. An aliquot of the primary stock solution was diluted to a concentration of 500 ng/mL with the mobile phase, and the samples were analyzed using the proposed method. The same procedure was followed for the tablets of 200 and 400 mg strength.

An aliquot of gatifloxacin injection (10 mg/mL strength) equivalent to 5 mg of drug was taken and diluted in the mobile phase to get a 50 µg/mL concentration primary stock. The rest of the sample preparation was the same as used for tablets, and the samples were then analyzed.

An aliquot of gatifloxacin ophthalmic solution (0.3%, w/v) equivalent to 1.5 mg of drug was taken and diluted in the mobile phase to get a 15 µg/mL concentration of primary stock solution. An aliquot of the primary stock solution was diluted to a concentration of 300 ng/mL with the mobile phase, and the samples were analyzed.

Twenty inserts were weighed and pulverized (the average insert weight was 10.32 mg). An amount of the powder equivalent to 150 µg of gatifloxacin was taken and sonicated with the mobile phase for 5 min. The solution was diluted suitably to prepare a 15 µg/mL concentration. The rest of sample preparation was the same as used for the ophthalmic solution, and the samples were analyzed. Five replicates were prepared in every case.

### Results and Discussion

#### Mobile phase composition optimization

In the mobile phase containing the citric acid buffer (pH 3.5) and ACN (50:50%, v/v), the retention time and asymmetric factor of the drug were found to be 3.6 and 1.83 min, respectively. The addition of ion-pairing reagents (SDS and TBAHS) to this mobile phase increased the retention time of the drug and reduced the asymmetric factor. When TBAHS concentration was changed from 0 to 20mM, keeping the SDS concentration at 10mM, the retention time decreased from 7.35 to 4.04 min and the asymmetric factor decreased from 1.43 to 1.22. However, the opposite effect was observed when the SDS concentration was varied (0 to 20mM) by keeping the TBAHS concentration at 10mM. The retention time increased from 2.24 to 6.22 min, and the asymmetric factor decreased from 1.38 to 1.18. Based on these experiments, the optimum peak parameters were obtained with 10mM SDS and 10mM TBAHS. The retention time of gatifloxacin was decreased from 11 to 4.6 min with a change in the concentration of ACN from 40% to 50% in the mobile phase (with 10mM SDS and 10mM TBAHS); however, there was no effect on the area and asymmetric factor of the drug. The retention time, area, height, and asymmetric factor of the drug was not affected with a change in the pH of the mobile phase (with 10mM SDS and 10mM TBAHS) from 3 to 4. Incorporation of TEA, methanol, and

#### Table II. Accuracy and Precision Data for the Developed Method. Each Determination is the Result of Six Separate Determinations

<table>
<thead>
<tr>
<th>Level</th>
<th>Predicted con. (µg/mL)*</th>
<th>Mean % Recovery (± SD)</th>
<th>Accuracy (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean (± SD)</td>
<td>% RSD</td>
</tr>
<tr>
<td>LQC</td>
<td>73.67 - 76.77</td>
<td>75.24 ± 0.95</td>
<td>1.26</td>
</tr>
<tr>
<td>MQC</td>
<td>381.94 - 405.18</td>
<td>398.24 ± 5.54</td>
<td>1.39</td>
</tr>
<tr>
<td>HQC</td>
<td>887.38 - 912.94</td>
<td>902.55 ± 5.87</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Predicted concentration of gatifloxacin was calculated by linear regression equation.
† Accuracy is given in relative error % (= 100 × [(predicted concentration – nominal concentration)/nominal concentration]).
phosphate buffer lowered response and increased the asymmetric factor. Thus, the final optimized mobile phase consisted of 25mM citrate buffer, 10mM SDS, and 10mM TBAHS with 48% ACN at pH 3.5.

**Calibration curve**

Different concentrations and their corresponding area are shown in the Table I. At all the concentration levels, the standard deviation (SD) of the area was low and the RSD did not exceed 1.96%. Chromatograms of three different concentrations are shown in Figure 1. The predicted concentrations nearly matched the nominal concentration. The linearity range in the selected mobile phase was found to be 50–1000 ng/mL. According to a linear regression analysis, the slope (± standard error) and intercept (± standard error) were found to be 105.5 (± 0.2) and −695.8 (± 108.3), respectively. These mean values were found to be within the 95% confidence limits (confidence limits of the slope were 105.1 to 105.9, confidence limits of the intercept were −910.7 to −480.9). Goodness of fit of regression equation was supported by a high regression coefficient value (0.9996), low standard error of estimate (6.73), low mean sum of the squared residuals value (44.87), and low calculated F-value [calculated $F(5,35)$ = 1.30 and critical $F$-value = 2.37 at $P = 0.05$ level of the significance]. Lower values of parameters like the standard error of the slope, intercept, and estimate indicated high precision of the proposed method.

**Analytical validation**

The blank samples of excipients did not show any interference near the drug peak. Figure 2 shows the chromatograms of gatifloxacin with hydroxypropyl methylcellulose in 1:50 proportion and its blank, and this excipient did not interfere with the drug peak. Similar results were observed with all other excipients (chromatograms not shown). In the presence of excipients, peak characteristics of the drug (retention time, area, and asymmetric factor) were not affected. From all the excipient–drug combination studies, the mean % recovery (± SD) value was found to be 101.16% (± 1.72). This indicated that there is no significant interference of excipients in the estimation of the drug by the proposed method. Therefore, the proposed method was specific and selective for the drug.

All three concentration levels showed an accuracy ranging from −0.44% to 0.33% (Table II). The high (nearly 100%) mean % recovery values and their low SD values (SD < 1.5) represented the accuracy of the method. In the standard addition method, the mean percentage analytical recoveries (± SD) for 50, 100, 250, and 500 ng/mL concentrations were found to be 101.88 (± 1.81), 99.39 (± 0.87), 101.48 (± 0.96), and 100.41 (± 0.66), respectively. This result revealed the validity and reliability of the proposed method.

In a repeatability study, the RSD ranged from 0.65% to 1.39% (Table II). RSD values were significantly low for intermediate precision, and intra-day variation was not more than 1.47%, though inter-day variation was less than 1.11% (Table...
Lower RSD values indicated the repeatability and intermediate precision of the method. LOD and LOQ were found to be 6.50 and 17.38 ng/mL, respectively. Figure 3 shows the chromatogram of gatifloxacin having a concentration at an LOQ of 17.4 ng/mL. Upon repeated injections, the characteristics of the drug peak (retention time, area, and asymmetric factor) were not affected. The mean % recovery (± SD) of the drug at LOQ was found to be 102.33% (± 2.65), representing the accuracy and precision of the method. Robustness was found to be very high, as variation of pH of the selected media by ± 1 did not have any significant effect on retention time (5.20 ± 0.04), asymmetric factor (1.22 ± 0.02), and % drug recovery (98.55 ± 2.30). Different concentrations of bench-top gatifloxacin solutions and stock solutions of gatifloxacin showed RSD values less than 2.56%, indicating the robustness of the proposed method. These solutions exhibited no change in chromatographic characters (retention time, asymmetric factor, and area) at least until 36 h at room temperature. During this period, no extra peaks were observed in the chromatograms at all concentrations.

**Estimation of formulations**

The proposed method was evaluated by estimation of gatifloxacin in pharmaceutical formulations. The assay values of gatifloxacin for different formulations ranged from 99.12% to 102.57% with SD not more than 2.00. Assay values of formulations were very close to the label claim. This indicated that the interference of excipient matrix was insignificant in estimation of gatifloxacin by the proposed method (Table IV). This result supports the applicability of this method to a variety of formulations.

**Conclusion**

Validation parameters of the proposed ion-pair RP-HPLC method proved high sensitivity, accuracy, precision, and robustness. The peak asymmetry and sensitivity of gatifloxacin was improved. LOQ of the proposed method was lower than the reported HPLC methods (13,14). The sample recoveries in all formulations were in good agreement with their respective label claims, indicating the non-interference of excipients in the estimation. This method can be used for routine analysis of gatifloxacin in bulk, pharmaceutical formulations, and for dissolution studies of oral and ophthalmic formulations.

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References


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