Method Validation for HPLC Assay of 7-Chloro-1-cyclopropyl-fluoro-1,4-dihydro-4-oxo-1,8-naphthylidine-3-carboxylic acid

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Gemifloxacin (Factive, LB20304a), a new chemical entity developed by LG Life Science Ltd. and approved by FDA on April 2003, is a broad-spectrum fluoroquinolone antibiotic used to treat respiratory infections.1 It was synthesized by the coupling reaction of the two key intermediates, 7-chloro-1-cyclopropyl-fluoro-1,4-dihydro-4-oxo-1,8-naphthylidine-3-carboxylic acid (QN09) and 3-aminomethyl-4-hydroxypyrrolidine (AM19), as shown in Figure 1.

Recently, we have developed a new QN09 synthetic process that is more cost-effective, operationally simpler than the previous one. The QN09 has two impurities and three intermediates that were not found in QN09 synthesized by the previous process. The impurities and intermediates were not separated completely from QN09 by using high performance liquid chromatographic (HPLC) separation conditions that was applied to analysis of QN09 produced by the previous synthetic process. The report on the previous analytical method and its validation is registered on the LG Life Science’s Drug Master File of Gemifloxacin.2

The objective of this study is to develop and validate the analytical method by which QN09 is separated and determined from the impurities and intermediates produced by the new synthetic process.

The new analytical method for the assay of QN09 has baseline separation with its degradation products, the new impurities and the intermediates with the resolution of 8 and the number of theoretical plates of > 20000. The method validation was done by evaluating specificity, linearity, accuracy, precision (i.e., repeatability and intermediate precision), robustness and solution stability complying with the International Conference on Harmonization (ICH) guideline Q2(R1).3

The new assay of QN09 was applied for the production of FACTIVE. The validation result will be reported to FDA.

![Figure 1. Synthesis of Gemifloxacin.](image-url)
Identification of QN09 and Impurities by LC-MS.

Figure 2 shows LC-MS analysis of QN09 sample. From the total ion chromatogram and MS spectra, QN09 and two impurities were identified: QN09 (m/z 282) at 27.8 min, two impurities (m/z 319 and m/z 291) at 20.2 and 25.5 min, respectively.

Development of HPLC Separation Condition of QN09 from Impurities.

HPLC separation conditions for determination of QN09 produced by the previous synthetic process did not give the baseline separation of QN09 from impurities produced by new process (Figure 3(a)). Luna C18 column showed the baseline separation among QN09 and the impurities as well as between two impurities as shown in Figure 3(b). From the comparison between separations in Figure 3(a) and 3(b), it was supposed that silica based column, Luna C18, gives better separation of QN09 from the impurities similar to QN09 structurally than polymer based column, Capcell Pak C18.

Specificity.

The homogeneity of QN09 peak was checked to confirm the specificity of the peak at retention time of 23.6 min (Figure 4). The comparison of spectra obtained from the apex and the other points at half of QN09 peak height gave that the peak was not interfered by any impurities because the three spectra were similar to each other (Figure 5).

The stress testing of QN09 sample was done by analyzing...
its degradation products. The four stress conditions were: 0.2 M HCl (acid), 0.2 M NaOH (base), 10% (v/v) H$_2$O$_2$ at 70 °C (oxidant) and sunlight (photo) according to ICH Q1A. The stress conditions were determined by pre-experiment. QN09 was degraded to four products under the conditions.

No significant degradations were observed within 24 hours in the sample stressed by 0.2 M HCl at 70 °C. Base stress testing gave a degradation product with 20% of peak area ratio at 4.3 min. Oxidant stress testing gave a degradation product with 62% of peak area ration at 13.6 min. Photo stress testing gave a degradation product with 45% of peak area ratio at 8.0 min. Figure 6 showed the baseline separation of QN09 and the degradation products resulting from each stress.

The specificity was also checked using a QN09 sample spiked with all its intermediates and impurities. Figure 7 showed that all peaks were separated completely.

**Linearity.** Linearity was investigated at eight levels in the range from 0.026 to 0.859 mg/mL (w/v) of QN09 concentration. Three replicates were performed at each level. The calibration curves were obtained with the average of peak area ratios of three replicates. The results showed a good linearity with the calculated correlation coefficient of 0.9999 (Figure 8).

**Accuracy.** The accuracy of the method was evaluated at three concentration levels of QN09. The accuracy (recovery) was determined using the sample spiked with standard solution of QN09. The recovery of QN09 was 100.6 ± 0.7% in the range between 0.240 and 0.479 mg/mL (w/v) (Table 1).

**Precision.** For the evaluation of precision, repeatability, intermediate precision, and reproducibility were investigated using samples at the concentrations ranged from 0.447 to 0.667 mg/mL (w/v) of QN09.

Repeatability, the intra-day variation in Daejeon (R&D site), was 0.1% of relative standard deviation (RSD) value. The intermediate precisions, which were inter-day and
different instrument variations, were 0.1% of RSDs in mobile phase. MeCN: water: TF A (34 : 66 : 0.1, v:v:v); Detector wavelength: 266 nm; Flow rate: 1.0 mL/min; temperature: 30°C.

Table 3. Robustness evaluation of the HPLC method for the determination of QN09

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Ratio</th>
<th>Resolution</th>
<th>Retention Time (min)</th>
<th>Theoretical Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luna C18</td>
<td>Acetonitrile/Water</td>
<td>30/70</td>
<td>8.68</td>
<td>21117</td>
</tr>
<tr>
<td>Capcell Pak C18</td>
<td>34/66</td>
<td>9.30</td>
<td>13.9</td>
<td>18662</td>
</tr>
<tr>
<td>Shodex ODP 60-5E</td>
<td>40/60</td>
<td>9.14</td>
<td>32.0</td>
<td>5523</td>
</tr>
<tr>
<td></td>
<td>Trilisobacetic Acid Ratio</td>
<td>0.05</td>
<td>8.45</td>
<td>21451</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>9.41</td>
<td>21811</td>
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<td>7.96</td>
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</table>

The variations of method parameters have an effect on system suitability specifications (resolution, number of theoretical plates, and retention time). The variation parameters are column type, mobile phase ratio, and column temperature.

From Table 3, Luna C18 column showed higher values of resolution and number of theoretical plates than Capcell Pak C18 and Shodex ODP 60-5E columns.

The ratio of organic solvent concentration and the column temperature had an effect on resolution, number of theoretical plates, which all of were above 8 and 20000 (the USP mandated limit), respectively.

**Robustness.** The aim of robustness test is to see if the variations of method parameters have an effect on system suitability specifications (resolution, number of theoretical plates, and retention time). The variation parameters are column type, mobile phase ratio, and column temperature.

**Stability of the Sample.** The stability study of QN09 in sample solution at analyte concentration was done to confirm the stability during the analysis after dissolving the samples. The sample solutions at 0.6 mg/mL (w/v) were stored in an autosampler at room temperature for 5 days. The RSD value of 0.4% indicated that QN09 was stable in solution for 5 days.

To determine the storage period of QN09 powder, the stability study was conducted at 25°C and relative humidity 60% complying with ICH Guide Q1A. The testing points for stability study were every 3 months over the first year and every 6 months over the second year. As the result of the stability study, it was confirmed that new QN09 is stable within 24 months (data not shown here).

**Conclusions**

The HPLC analysis method was developed to separate QN09 from impurities. Linearity, precision, accuracy, specificity and robustness were investigated to validate the HPLC method for the assay of QN09. The result of the validation show that the assay of QN09 was suitable for the quality control of QN09 as an intermediate for the production of the drug product, FACTIVE. The validation result will be reported to FDA.

**References**