# 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 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-hydroxy-pyrrolidine (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.

## Experimental

**Standard and Materials.** QN09 was obtained from Korea Fine Chemicals Co. (Yeosu, Korea). Its reference standard and all the synthetic intermediates were provided by process development group of Research & Development, LG Life Sciences Ltd. (Daejeon, Korea). QN09, standard and intermediates were dissolved in acetonitrile:water (9 : 1, v:v) mixture. Acetonitrile (MeCN) was purchased from J. T. Baker (Phillipsburg, NJ08865, USA). Trifluoroacetic acid (TFA) was obtained from Aldrich (St. Louis, MO63103, USA). Hydrochloric acid and sodium hydroxide were purchased from Fluka (USA). Purified water was prepared by Milli-Q water purification system (Millipore, MA, USA).

**Instrumental Analysis.**

**HPLC Analysis:** Two HPLC systems were used for the chromatographic analysis: Waters 2690 HPLC system equipped with Waters 996 photodiode array detector (PDA) under the control of Millennium 3.20 software and Agilent 1100 series HPLC system equipped with diode array detector (DAD) under the control of Chemstation 8.03 software.

The separation of QN09 from impurities and intermediates was done on Luna $C_{18}$ column (4.6 mm ID × 250 mm L, 5 $\mu$m, Phenomenex) with mobile phase of mixture of MeCN, purified water and TFA (34 : 66 : 0.1, v/v/v) at detection wavelength of 266 nm, flow rate of 1.0 mL/min and temperature of 30 °C. Capcell pak C18 column (4.6 mm ID × 250 mm L, 5 $\mu$m, Shiseido) and Shodex ODP 60-5E (4.6 mm ID × 250 mm L, 5 $\mu$m, Shodex) column were used for the robustness study.

**Liquid Chromatography-Mass spectrometry (LC-MS) Analysis:** Agilent 1100 series HPLC system interfaced with Finnigan LCQ ion-trap mass spectrometer (San Jose, CA, USA) with an atmospheric pressure chemical ionization

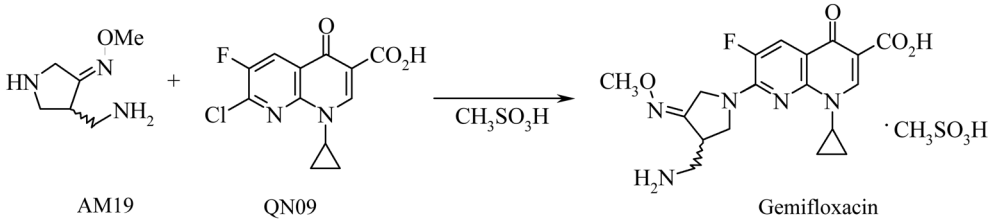


**Figure 1**. Synthesis of Gemifloxacin.

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(APCI) source of positive mode was used to identify QN09, impurities and intermediates. Xcalibur software (rev. 1.2, Finnigan) was used to control mass spectrometer and process data. Liquid chromatographic separation was done by using conditions that were applied to HPLC-DAD analysis. Mass spectrometric conditions are: sheath and auxiliary gas $N_2$; sheath gas flow-rate 80 mL/min; auxiliary gas flow-rate 10 mL/min; capillary voltage 3.5 eV; scan range *m/z* 50-1500; vaporizer temperature 200 °C; capillary temperature 200 °C.

## Results and Discussion

**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

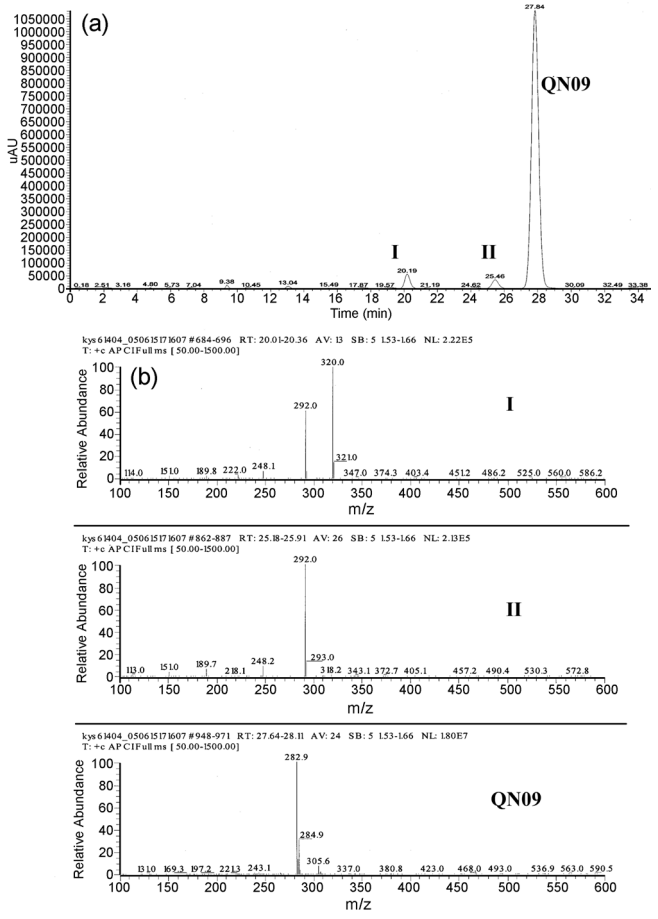


**Figure 2**. LC-MS analysis of QN09 spiked with impurity **I** and impurity **II**. (a) Total Ion Chromatogram of QN09 sample. (b) Mass spectra of QN09, impurity **I** and impurity **II**.

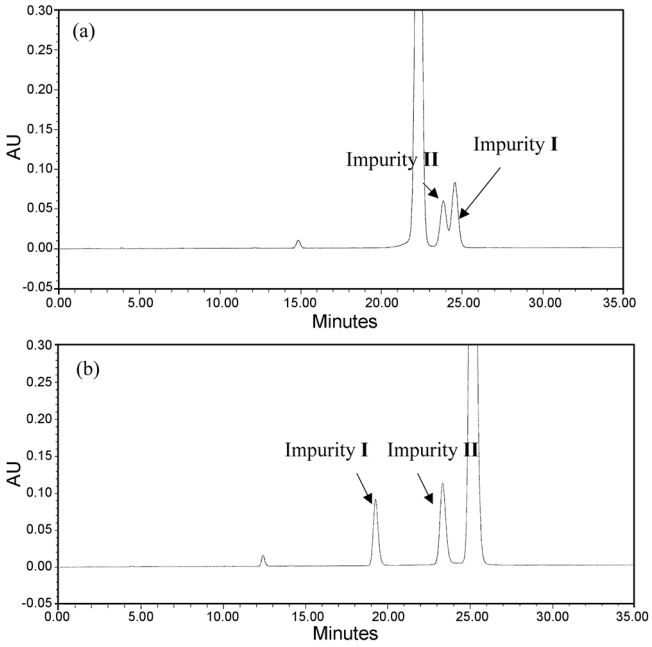


**Figure 3**. HPLC-DAD analysis of QN09 spiked with impurity **I** and impurity **II**. (a) Chromatogram by the previous analytical condition. Column: Capcell pak $C_{18}$, 4.6 mm × 250 mm, 5 $\mu$m; mobile phase: MeCN: water: TFA (30 : 70 : 0.1, v:v:v); detector wavelength: 266 nm; flow rate: 1.2 mL/min; Temperature: 35 °C. (b) Chromatogram by the new analytical condition. Column: Luna $C_{18}$, 4.6 mm × 250 mm, 5 $\mu$m; mobile phase: MeCN: water: TFA (34 : 66 : 0.1, v:v:v); detector wavelength: 266 nm; flow rate: 1.0 mL/min; Temperature: 30 °C

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,[4] Luna C18, gives better separation of QN09 from the impurities similar to QN09 structurally than polymer based column,[5] 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

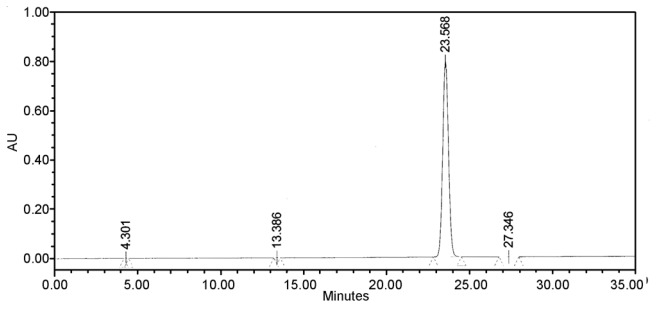


**Figure 4**. HPLC-PDA chromatogram of QN09 (Wavelength 266 nm). Column: Luna $C_{18}$, 4.6 mm × 250 mm, 5 $\mu$m; mobile phase: MeCN: water: TFA (34 : 66 : 0.1, v:v:v); detector wavelength: 266 nm; flow rate: 1.0 mL/min; Temperature: 30 °C

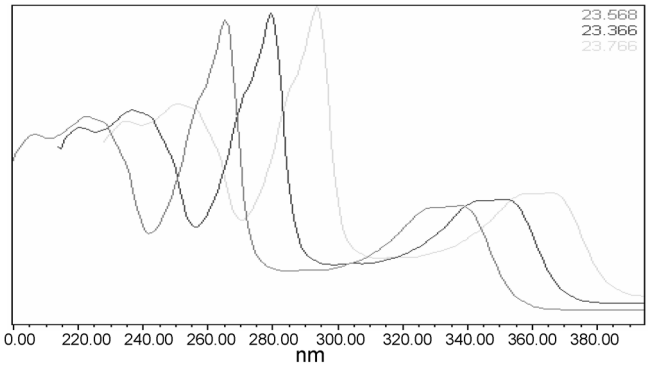


**Figure 5**. UV spectra for the apex and two points corresponding to half the height of the peak for QN09.

its degradation products. The four stress conditions were: 0.2 M HCl (acid), 0.2 M NaOH (base), 10% (v/v) $H_2O_2$ at 70 °C (oxidant) and sunlight (photo) according to ICH Q1A.[6] The stress conditions were determined by pre-experiment (pre-experiment data not shown here.). 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

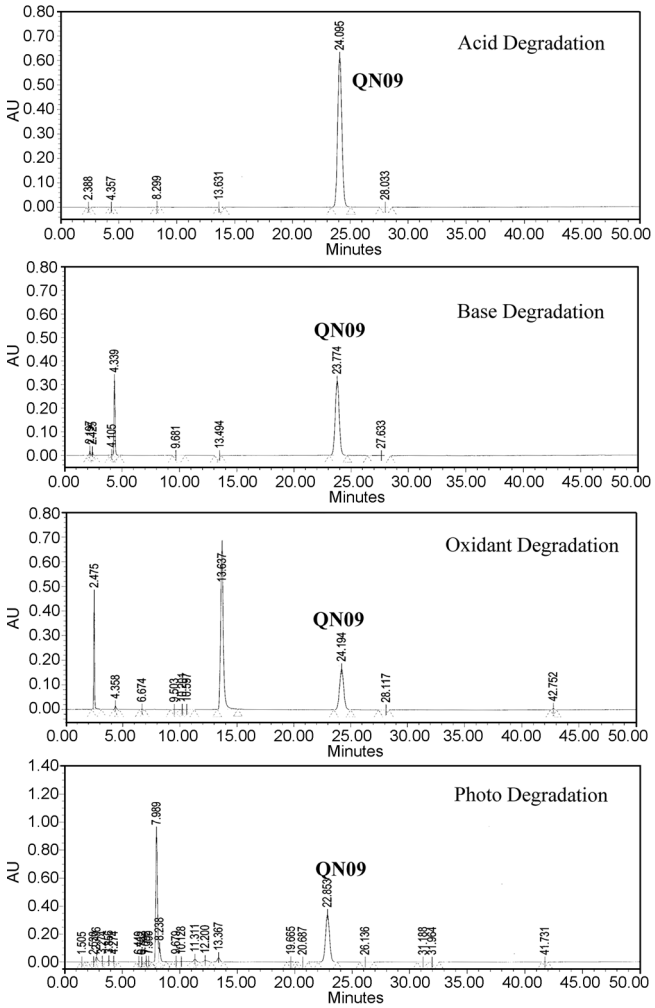


**Figure 6**. HPLC-PDA analysis of QN09 samples degraded by four stresses.

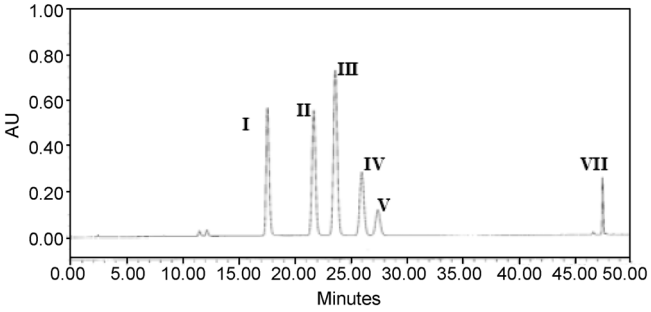


**Figure 7**. HPLC-PDA chromatogram of QN09 spiked with impurities and intermediates: **I** and **II** impurity, **III** QN09, **IV** QN08, **V** QN16, **VII** QN07.

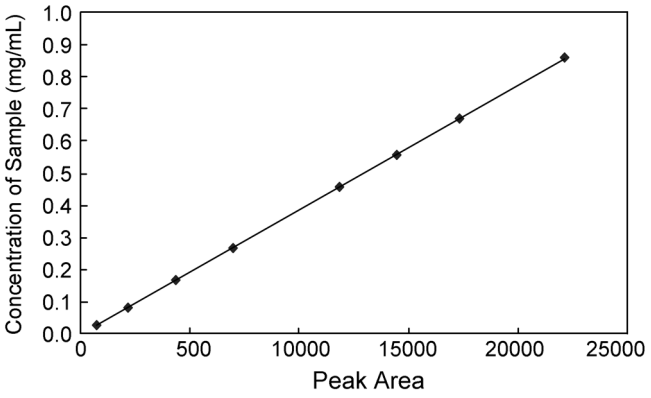


**Figure 8**. Linearity of QN09 produced by synthetic process.

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

**Table 1**. Recovery of QN09

| Spiked Concentration of QN09 (mg/mL) | Calculated Concentration of QN09 (mg/mL) | % Recovery |
|---|---|---|
| 0.2395 | 0.2381 | 99.4 |
| | 0.2398 | 100.1 |
| | 0.2435 | 101.7 |
| 0.3592 | 0.3619 | 100.8 |
| | 0.3619 | 100.8 |
| | 0.3599 | 100.2 |
| 0.4790 | 0.4806 | 100.3 |
| | 0.4844 | 101.1 |
| | 0.4834 | 100.9 |
| | Average | 100.6 |
| | R.S.D[a] | 0.7 |

[a]R.S.D: Relative Standard Deviation

**Table 2**. Precision of QN09

| n | Repeatability[a] (%) | Intermediate Precision (after 7 days) | | Reproducibility Iksan Labs (%) |
|---|---|---|---|---|
| | | Waters LC (%) | Agilent LC (%) | |
| 1 | 99.5 | 99.5 | 99.4 | 99.8 |
| 2 | 99.5 | 99.5 | 99.4 | 99.0 |
| 3 | 99.4 | 99.4 | 99.4 | 99.7 |
| 4 | 99.4 | 99.4 | 99.5 | 99.3 |
| 5 | 99.4 | 99.4 | 99.6 | 99.6 |
| 6 | 99.5 | 99.5 | 99.6 | 99.6 |
| Average | 99.5 | 99.5 | 99.5 | 99.5 |
| RSD[b] | 0.1 | 0.1 | 0.1 | 0.3 |

[a]The values were determined using Waters LC in Daejeon. [b]RSD: Relative Standard Deviation

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

| | Chromatographic Change Factor | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Column Type[a] | | | Acetonitrile/Water[b] Ratio | | | Trifluoroacetic Acid[c] Ratio (%) | | | Column Temperature[d] (°C) | | |
| | Luna | Capcell Pak C18 | Shodex ODP 60-5E | 30/70 | 34/66 | 40/60 | 0.05 | 0.10 | 0.15 | 25 | 30 | 35 |
| Resolution | 8.68 | 5.30 | 7.68 | 8.45 | 9.41 | 7.96 | 9.14 | 9.03 | 8.90 | 10.57 | 9.70 | 8.93 |
| Retention Time (min) | 25.2 | 13.9 | 32.0 | 39.4 | 26.6 | 16.1 | 24.5 | 25.2 | 25.2 | 28.1 | 26.4 | 25.0 |
| Theoretical Plates | 21117 | 15662 | 5523 | 21451 | 21811 | 22402 | 21521 | 21134 | 21496 | 21633 | 21601 | 22027 |

[a]Mobile phase: MeCN: water: TFA (34 : 66 : 0.1, v:v:v); Detector wavelength: 266 nm; Flow rate: 1.0 mL/min; Temperature: 30 °C. [b]Column: Luna $C_{18}$; Mobile phase was added 0.1 % TFA; Detector wavelength: 266 nm; Flow rate: 1.0 mL/min; Temperature: 30 °C. [c]Column: Luna $C_{18}$; MeCN/Water ratio was 34/66 (v/v); Detector wavelength: 266 nm; Flow rate: 1.0 mL/min; Temperature: 30 °C. [d]Column: Luna $C_{18}$; Mobile phase: MeCN: water: TFA (34 : 66 : 0.1, v:v:v); Detector wavelength: 266 nm; Flow rate: 1.0 mL/min

different instrument variations, were 0.1% of RSDs in Daejeon (R&D site). The reproducibility was 0.3% of RSD in Iksan (commercial site).

**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.

From Table 3, Luna $C_{18}$ 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 not an effect on resolution, number of theoretical plates, which all of were above 8 and 20000 (the USP mandated limit[7]), respectively.

**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.[8] 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.[5] 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.

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