Validated Stability-Indicating RP-HPLC Method for the Determination of Norfloxacin in Pharmaceutical Dosage Form

Sirajunisa Talath¹* and Syeda Humaira²

¹Department of Pharmaceutical Chemistry, RAK College of Pharmaceutical Sciences, RAKMHSU, POB 11172, Ras Al Khaimah, UAE
²Department of Pharmaceutical Chemistry, Luqman College of Pharmacy, Gulbarga India

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Corresponding author: Sirajunisa Talath
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Abstract
The objective of this work was to develop a simple, sensitive, accurate, precise and reproducible high performance liquid chromatography (HPLC) method for the determination of norfloxacin in pharmaceutical dosage forms. Shimadzo Prominance model L20 AD HPLC system equipped with SPD 20A UV-Vis detector was used for the analysis. The separation was done on RESTEX allure C18 column (3 μm, 15 cm × 4.6 mm), for an isocratic elution a mixture of methanol and water (60:40, v/v) mobile phase at a wavelength of 254 nm. The flow rate was 1.0 mL/min. The RP-HPLC method developed for analysis of norfloxacin was validated with respect to specificity, selectivity, linearity, accuracy, precision and robustness as per the ICH guidelines. The retention time of norfloxacin was 7.5 min. The linearity was established over the concentration ranges of 50-350 μg/mL with correlation coefficients (r²) 0.999. The percentage accuracy of norfloxacin ranged from 99.76-101.66%. The relative standard deviation values for intra-day and inter-day precision was lower than 2.0% and the assay result was found to be 100.65 %. Norfloxacin was subjected to stress conditions such as neutral, acidic, alkaline, oxidation and photolysis degradations as per ICH guidelines. The degradation studies revealed that the drug was found to degrade maximum (1.67%) in alkaline degradation conditions and was highly resistant towards neutral, acidic, oxidative and photolytic degradation conditions.

Keywords: Norfloxacin, Validation, Stability-indicating, stress degradation, ICH guidelines.

Introduction

Norfloxacin chemically known as 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline-3-carboxylic acid is a broad spectrum synthetic fluoroquinolone antibiotic (Fig. 1). Norfloxacin is the first synthetic second-generation fluoroquinolone antimicrobial drug. It was developed for use in human and veterinary medicine [1]. Norfloxacin, occasionally used to treat common, as well complicated urinary tract infections, exhibits a broad spectrum of activity against Gram-positive and Gram-negative bacteria [2-4]. Chemically, it is 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid (Figure 1). The mechanism of the bacterial effect of
norfloxacin is based on the primary target in bacterial enzyme DNA gyrase and topoisomerase II and IV. Inhibition of the activity of these enzymes disables DNA replication which in turn, inhibits bacterial replication [5, 6].

International Conference on Harmonization (ICH) has made stability-indicating assay method (SIM) for every drug candidate mandatory as it helps in establishing the intrinsic stability of the drug and also assures changes in identity, purity and potency of the product on exposure to various conditions. Hence, it was meticulous for us to study the force degradation studies of norfloxacin by subjecting it to stress conditions (viz. acidic, alkaline, oxidative, dry heat, and photolytic stress) as described in the ICH guidelines. [7, 8]

The literature reported analytical methods used in the quantitative estimation of norfloxacin alone or in combination with other drugs reported includes spectrophotometric methods, liquid-liquid micro extraction, liquid chromatography coupled with - tandem mass spectrometry, fluorescence detection, and UV detection. However, very few reported the estimation of norfloxacin from marketed formulations. [9–23]

From the literature review, it was professed that various analytical methods are accessible for analyzing norfloxacin in pharmaceutical preparations. Thus, the objective of our study was to develop a simple, precise, specific, accurate, cost-effective validated RP-HPLC method according to USP and ICH guidelines for the quantitative estimation of norfloxacin in presence of its degradation products or other pharmaceutical excipients. The analytical method developed was applied to study the stress degradation studies of norfloxacin as suggested by ICH guidelines. [24, 25, 7, 8]

Materials And Methods

Reagents And Chemicals

Methanol and water used were of HPLC grade (Fisher Scientific, UK). Sodium hydroxide (NaOH), hydrogen peroxide (H₂O₂) and hydrochloric acid (HCl) were obtained from Scharlau, Spain. Norfloxacin standard (purity 100%) was kindly gifted by Julphar Gulf Pharmaceuticals, Ras Al Khaimah. All the chemicals procured were of analytical grade and used as received.

HPLC Apparatus And Conditions

Chromatographic separation was accomplished using the instrument Shimadzo Prominance model L20 HPLC system equipped with SPD 20A prominence UV-Vis detector, RESTEX allure C18 (3 μm, 15 cm × 4.6 mm) column. Isocratic elution was performed using the solvent system as a mixture of methanol and water (60:40, v/v) and UV detection at 254 nm. The overall run time of the analysis was 10 minutes and the flow rate was 1.0 mL/min. 20 μL of sample was injected into the HPLC system. All the analyses were carried out at room temperature. Results were acquired and processed by Shimadzu LC Solution software.

Method Development

Preparation of the mobile phase: The mobile phase was prepared by mixing methanol and water (60:40, v/v). The solution was filtered through 0.45μm membrane filter and sonicated for about 15 minutes.

Preparation of Standard Solution: Standard stock solution of norfloxacin was prepared using the diluent mixture [methanol and water (70:30)] to obtain a concentration of 1mg/mL. The procedure involved accurately weighed 10mg of norfloxacin standard sample and transferred into a 10ml volumetric flask, dissolved in 5ml of diluent. The resultant solution was sonicated for about 5 minutes to dissolve the drug completely and finally made the volume up to 10 ml with methanol to get the primary stock solution of 1mg/mL (1000μg/mL). Further, sample solutions were prepared by appropriate dilution of standard solutions with diluent. The solution was mixed well and filtered through 0.45μm membrane filter. Aliquots of the suitable norfloxacin working standard solutions were transferred
into a series of 10 mL volumetric flasks so that the final concentration was in the range of 50-350 \( \mu \)g/mL. The working standard solution (300 \( \mu \)g/mL) was prepared by taking 3 mL of stock solution in 10ml volumetric flask and diluted up to 10ml with methanol. The solution was mixed well and filtered through 0.45 \( \mu \)m membrane filter.

**Analytical Method Validation**

In the present study, analytical method developed for the estimation of norfloxacin was validated with respect to system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and ruggedness. Later the modified method was used to estimate the percentage content of norfloxacin in pharmaceutical cream containing norfloxacin (3% w/w). [24, 25]

**System Suitability**

The system suitability studies were performed to confirm that the resolution and reproducibility of the chromatographic system is adequate for the analysis. The assessment for the suitability of the system was performed using six (6) norfloxacin replicas at concentration of 300 \( \mu \)g/mL. Various parameters assessed included repeatability, retention time, peak area, capacity factor, tailing factor, theoretical plates of the column. Results of the analysis are summarized in Table 1. A typical chromatogram of norfloxacin is shown in Figures 3A-B.

**Linearity**

Eight levels of standard calibration solutions of norfloxacin were prepared from the stock solutions in the concentration range of 50-350 \( \mu \)g/mL to comprehend the expected concentration in the measured sample and the calibration graph (concentration vs. peak area) was constructed. The analytical curve generated was evaluated on three different days. The calibration curve of norfloxacin is reported in Figure 2 and the data for linear regression studies is shown in Table 2.

**Sensitivity**

The sensitivity of norfloxacin was determined in terms of Limit of quantification (LOQ) and Limit of detection (LOD) as per the USP guidelines.\(^3\)

**Precision**

Precision was determined by repeatability (intraday precision) and intermediate precision (interday precision) of standard and sample solutions of norfloxacin. Precision was determined in six replicates of norfloxacin solution in the concentration range 50-350 \( \mu \)g/mL on the same day (intra-day precision) and daily for 6 times over a period of three days (interday precision). The results were expressed as %RSD of the measurements.

**Intra-Day Precision**

In the intra-day studies, six replicate injections of standard solutions of norfloxacin in the concentration (50-350 \( \mu \)g/mL) were injected into the HPLC system at different time intervals within a day. % RSD was calculated for each analysis was calculated and summarized in table 3.

**Inter-Day Precision**

In the inter-day studies, six injections of standard solutions of norfloxacin in the concentration (50-350 \( \mu \)g/mL) were injected into the HPLC system at different time intervals over a period of three days. % RSD was calculated for each analysis was calculated and summarized in table 3.

**Accuracy**

Accuracy of the method was determined by calculating recoveries of drug by method of standard addition. Known amount of standard drug corresponding to 50%, 100%, and 150% of the label claim was added to prequantified sample solution and the amounts of drug were estimated by measuring peak areas and the results of the study is represented in the Table 4.

**Robustness**

The robustness as a measure of method capacity to remain unaffected by slight
deliberate changes in chromatographic conditions. The chromatographic parameters selected were the effect of methanol in the mobile phase composition (63 and 67%), flow rate (0.8 and 1.2 mL/min) and wavelength (252 and 256 nm). Only one parameter was changed while the others were kept constant. Results of the study are summarized in Table 5.

Analysis of Marketed Formulation

Norfloxacin Cream (Norflo cream; Label claim 3 % w/w norfloxacin) was used to determine the drug content. 3 gm of the cream was accurately weighed and transferred carefully into 100 mL volumetric flask. The cream was dissolved in methanol by gentle heating. The solution was filtered through 0.22 μm millipore filter paper and volume was adjusted using methanol. The final concentration of working solution equivalent to 300 μg/mL was prepared by appropriate dilution in methanol. The resulting solution was filtered and subjected to chromatographic analysis in triplicate. Typical chromatograms for the formulation are shown in the figure 4A and the percent drug recovery data is summarized in Table 6.

Forced Degradation Solutions

Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. [7,8] Stability of norfloxacin was determined by subjecting it to oxidative, alkaline, acidic, neutral, and photolytic conditions in order to accelerate conditions auspicious for degradation. The stress solutions of norfloxacin at concentration of 300μg/mL were prepared from stock solution of 1 mg/mL using methanol and subjected to heating (80°C). Standard stress solutions norfloxacin was filtered through 0.45 μm membrane filter and injected into HPLC at regular time intervals. The HPLC chromatograms of the degradation studies are shown in Figures 5A-E and percent drug degraded is displayed in the table 7.

Neutral Degradation

Norfloxacin sample (300μg/mL) was treated with methanol for about 30 min in a thermostat maintained at temperature of 80 °C. Later cooled to room temperature and diluted with methanol, filtered through 0.45 μm membrane filter and injected into HPLC system. At regular time intervals, 20 μl of norfloxacin sample solutions were injected into the HPLC system and the chromatogram recorded is presented in Figure 5A.

Acidic Degradation

Acid degradation studies were achieved by treating norfloxacin solution (300μg/mL) with 0.1 N hydrochloric acid (0.1N HCl) for about 30 min in thermostat maintained at 80 °C. Later cooled to room temperature neutralized with 0.1N NaOH and diluted with methanol, filtered through 0.45 μm membrane filter paper and injected into HPLC system. The chromatogram recorded is presented in Figure 5B.

Alkaline Degradation

Alkaline degradation studies were performed by treating the norfloxacin solution (300μg/mL) with 0.1 N sodium hydroxide for about 30 min in a thermostat maintained at 80 °C. Later it was cooled to room temperature neutralized with 0.1N HCl, diluted with methanol and filtered through 0.45 μm membrane filter paper before injecting into the HPLC system. The chromatogram recorded is presented in Figure 5C.

Oxidative Degradation

Oxidative degradation was performed by treating norfloxacin solution (300μg/mL) with 3 % H₂O₂ for 30 min in a thermostat maintained at 80 °C. Later it was cooled to room temperature, diluted with methanol and filtered through 0.45 μm membrane filter before injecting into the HPLC system. The chromatogram recorded is shown in Figure 5D.

Photolytic Degradation

Norfloxacin was exposed to direct sunlight for 7 days. Stock solution of norfloxacin (1mg/mL) was prepared using the standard procedure described above. The solution obtained was
further diluted with methanol to obtain a concentration of 300 μg/mL and 20μL was injected into the HPLC system. The chromatogram recorded is shown in Figure 5E.

Results and Discussion

Method Development

The HPLC method carried out in the present experimental work was aimed at developing a new system capable of eluting resolving norfloxacin and its degradations products. Based on trial and error method, the mobile phase, which gave best possible separation and resolution, was selected and retention time was also taken into the consideration. During the development of this method, different compositions of mobile phase were tested. The best mobile phase was chosen after several trials with methanol, acetonitrile, water and acetic acid in various proportions. Finally, the mobile phase consisting of methanol and water, (60 : 40, v/v) was selected to achieve maximum separation and sensitivity. A flow rate of 1.0 ml/min gave an optimal signal to noise ratio with a reasonable separation time. Using RESTEX allure C18 column (3 μm, 15 cm × 4.6 mm), the retention time for norfloxacin observed to be 7.575 min, respectively. Total time of analysis was 10 min and the detection wavelength was found to be 254 nm.

System Suitability

The study was performed by collection of data from a standard solution containing 300μg/mL of norfloxacin that was injected six times of standard resolution solution. The parameters measured were tailing factor, capacity factor, theoretical plates, and retention time. % RSD for tailing factor was 1.595, the capacity factor was more than 2 (3.358 ± 0.513) and the theoretical plates were more than 2000 (2214.68 ± 0.19). The average of retention time was 7.65 minutes and peak area was 208786.95 ± 0.057. The results (Mean ± % RSD of six replicates) of the chromatographic parameters are shown in Table 1. Typical chromatograms of norfloxacin pure drug150 and 300 μg/mL is shown in the Figures 3A-B. The method was found to be precise and specific.

Method Validation

HPLC method was validated according to the International Conference on Harmonization Guidelines.[24, 25] The method was validated with respect to parameters including linearity, limit of detection (LOD), and limit of quantitation (LOQ), recovery, precision, accuracy, robustness, and specificity.

Linearity

Linearity for detector response was observed in the concentration range 50-350 μg/mL for norfloxacin. The calibration curve for norfloxacin was constructed with concentration against peak area (Figure 2). The linear regression data values are shown in Table 2. The regression equation for the calibration curve was found to be y = -6434.5+731.335x and the correlation coefficient (r2) of 0.999 was obtained. Good linearity was found between the peak area and analyte concentration.

Precision

Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (interday). The precision of the method was evaluated by performing six independent determinations of the standard norfloxacin solutions of six different concentrations (50-350 μg/mL) and calculating RSD (%). For day 1 (one) precision studies, the RSD (%) values for the six samples of norfloxacin was observed in the range of 0.54-0.76 while for day 3 (three) precision studies the RSD (%) range was 0.55-0.79. This shows that precision of the method is satisfactory as % relative standard deviation is not more than 2.0%. The results are depicted in Table 3.

Limit OF Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection and limit of quantitation for norfloxacin was calculated from the linearity data using relative standard deviation of the response and slope of the calibration
curve. By the analysis of samples with known concentrations of analyte and establishing the minimum level at which the analyte can be reliably detected and we found 5µg/mL of norfloxacin. Limit of quantification is the concentration that can be quantified reliably with a specified level of accuracy and precision. LOQ was found to be 25 µg/mL of norfloxacin. The results indicate that this method is sensitive.

Accuracy
To prove the accuracy of the proposed RP-HPLC method, recovery studies were accomplished by standard addition method at three different concentration levels (50%, 100% and 150%) summarized in table 4. Percent RSD for norfloxacin was found to be in the range 0.386-0.76 and the percentage recovery was 99.76-100.66%. The results of the recovery test indicate that the method is highly accurate.

Robustness
Robustness of the analytical method was determined by consistency of the peak height and peak shape with the deliberate small changes in the experimental conditions. Under all the deliberately altered chromatographic conditions (flow rate, mobile phase and wavelength), peaks were adequately resolved and elution orders remained unchanged which indicate that the developed method for norfloxacin is robust. The results are summarized in Table 5.

Analysis of Marketed Formulations
The proposed validated method was applied for the quantification of norfloxacin in norfloxacin eye cream (Norflox Cream, Label claim 3%w/w). The results of the assay are shown in Table 6 and HPLC chromatogram for the representative samples are presented in Figures 4A. The percentage recovery of the drug was found to be 100.65 %. The assay results indicate that the validated method was sensitive and specific for the quantitative analysis of norfloxacin in the marketed formulation.

Forced Degradation Studies
In order to evaluate the stability indicating properties of the developed method, forced degradation studies were carried out in accordance with ICH guidelines. [7, 8] The stability of norfloxacin was determined by exposing the pure sample to neutral, acidic, alkaline, oxidative and photolytic conditions in order to accelerate conditions favorable to degradation. The results and typical chromatograms of the degradation studies are displayed in the table 7 and figures 5A-E, respectively. During acid hydrolysis process, (0.1N HCl for 30 min), it was found that 0.27% of norfloxacin content was decreased, but there was no detectable degradation peak(s). The test samples submitted to alkaline condition (0.1N NaOH for 30 min) showed 1.67% degradation of norfloxacin content, and also there was no detectable degradation peak(s). In both cases, the peak purity was 99.99%. The samples in presence of neutral, oxidative and photolytic stress degradation conditions displayed the degradation percent for norfloxacin as 0.08 and 0.25, respectively. From the chromatograms for the degradation studies for norfloxacin, good selectivity and resolution of the compound and absence of degraded products seem to suggest that HPLC is a selective and specific method for the analysis of norfloxacin samples from stability studies.

| Sl. No | Parameters          | Value (Mean ± % RSD)* |
|--------|---------------------|-----------------------|
| 1      | Retention time      | 7.65 ± 0.64           |
| 2      | Peak area           | 208786.95 ± 0.057     |
| 3      | Tailing factor      | 1.0141 ± 1.595        |
| 4      | Theoretical plates  | 2214.68 ± 0.19        |
| 5      | Capacity factor     | 3.358 ± 0.513         |

* Mean and % RSD of six samples of norfloxacin
Table 2: Parameters of regression analysis data for norfloxacin

| Sl. No | Statistical Parameters       | Values                  |
|--------|-----------------------------|-------------------------|
| 1      | Concentration range         | 50-350\(\mu \text{g/mL}\) |
| 2      | Regression equation         | -6434.5+731.335x        |
| 3      | Correlation coefficient \((r^2)\) | 0.999                   |

Table 3: Results of Intraday and Interday precision studies for norfloxacin

| Concentration (µg/mL) | Day 1 | Day 2 |
|-----------------------|-------|-------|
|                       | Peak Area (Mean ± SD) | %RSD | Peak Area (Mean ± SD) | %RSD |
| 50.01                 | 33608.3 ± 213.13       | 0.63 | 33838.3 ± 223.64       | 0.66 |
| 100.11                | 65416.5 ± 353.27       | 0.54 | 65675.5 ± 381.86       | 0.58 |
| 150.09                | 98124.76 ± 742.37      | 0.76 | 98236.76 ± 779.05      | 0.79 |
| 200.03                | 139249.26 ± 853.55     | 0.61 | 139308.09 ± 917.97     | 0.66 |
| 250.1                 | 182910.98 ± 979.25     | 0.61 | 183115.98 ± 998.85     | 0.66 |
| 300.06                | 208774.77 ± 1069.08    | 0.54 | 208875.77 ± 1095.89    | 0.55 |

*Mean and % RSD of six samples of norfloxacin

Table 4: Results of accuracy studies for norfloxacin

| Amount added (µg/mL) | *Mean Peak area for norfloxacin ± SD | %RSD | *Amount recovered (µg/mL) | % Recovery |
|----------------------|---------------------------------------|------|---------------------------|------------|
| 150.09               | 98124.76 ± 742.37                     | 0.76 | 150.97                    | 100.59     |
| 300.06               | 208742.98 ± 1051.11                   | 0.50 | 302.03                    | 100.66     |
| 450.07               | 307932.29 ± 1189.36                   | 0.386| 449.03                    | 99.76      |

* Mean and % RSD of six samples of norfloxacin

Table 5: Results of Robustness studies for norfloxacin

| Condition                          | Modification | *Mean Peak area for norfloxacin ± SD | %RSD |
|------------------------------------|--------------|---------------------------------------|------|
| Mobile phase composition [Methanol and Water (60:40, v/v)] | 58:42        | 208778.98 ± 1043.15                   | 0.50 |
|                                    | 62:38        | 208806.98 ± 1019.1                    | 0.49 |
| Flow rate (1mL/min)                | 0.8 mL       | 208786.98 ± 1072.22                   | 0.51 |
|                                    | 1.2 mL       | 208781.23 ± 1051.11                   | 0.50 |
| Wavelength 254nm                   | 252 nm       | 208779.68 ± 1143.85                   | 0.55 |
|                                    | 256 nm       | 208812.98 ± 1163.89                   | 0.56 |

* Mean and % RSD of six samples of norfloxacin

Table 6: Determination of norfloxacin in semisolid dosage form

| Cream brand names of norfloxacin (3% w/w) | *Amount added (µg) | *Amount recovered (µg) | % Recovery |
|------------------------------------------|-------------------|------------------------|------------|
| Norflox Cream                            | 300.05            | 302.02                 | 100.65     |

Mean of six samples of norfloxacin
Table 7: Results of Stress degradation studies for norfloxacin

| Sl. No | Stress condition | *Mean Peak area for norfloxacin ± SD | % Drug recovered | % Drug degraded |
|--------|------------------|--------------------------------------|------------------|----------------|
| 1      | Neutral          | 208772.98 ± 1045.53                  | 100              | 0              |
| 2      | Acidic           | 208784.98 ± 1012.12                  | 99.73            | 0.27           |
| 3      | Alkaline         | 208776.53 ± 1053.19                  | 98.33            | 1.67           |
| 4      | Oxidative        | 208791.14 ± 1049.42                  | 99.92            | 0.08           |
| 5      | Photolytic       | 208778.50 ± 1051.33                  | 99.75            | 0.25           |

*Mean and standard deviation (SD) of six samples of norfloxacin

Figure 1: Chemical Structure of Norfloxacin

Figure 2: Calibration curve for norfloxacin (concentration range 50-350 µg/mL)
Figure 3: Typical chromatograms of norfloxacin pure drug: (A) (150 μg/mL); (B) (300 μg/mL).

Figure 4: Typical HPLC chromatograms for the formulations of norfloxacin (300 μg/mL): A. Norflox Cream.
Figure 5A: HPLC chromatogram of norfloxacin (300μg/mL) after exposure to neutral degradation.

Figure 5B: HPLC chromatogram of norfloxacin (300μg/mL) after exposure to acid hydrolysis (0.1 N hydrochloric acid for 30 min 80 °C).

Figure 5C: HPLC chromatogram of norfloxacin (300μg/mL) after exposure to alkaline hydrolysis (0.1 N sodium hydroxide for 30 min 80).
Figure 5D: HPLC chromatogram of norfloxacin (300μg/mL) after exposure to oxidative degradation (3 % H₂O₂ for 30 min in a thermostat maintained at 80 ºC).

Figure 5E: HPLC chromatogram of norfloxacin (300μg/mL) after exposure to photolytic degradation.

Conclusion
The proposed method for the determination of norfloxacin based on the RP-HPLC method with spectrophotometric detection was shown to be reliable, simple, accurate, sensitive and precise. The validated method could be successfully applied for the determination of norfloxacin in pharmaceutical preparation without interference from co-formulated drugs. The good validation criteria of the proposed method allow its use in quality control laboratories as an alternative to the official methods. The detection limit of the proposed method was found to be 5 μg/ml while the quantitation limit was 25 μg/mL. The results demonstrated the ability of the proposed method to be used as a stability-indicating HPLC method for the analysis of norfloxacin.

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