Development and Validation of a RP-HPLC Method for Simultaneous Determination of Levofloxacin and Moxifloxacin in Pharmaceutical Dosage Forms

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Abstract: A simple, fast and economic reversed phase high performance liquid chromatographic (HPLC) method has been successfully developed and validated for simultaneous determination of fluoroquinolone analogs namely levofloxacin and moxifloxacin in both pure form (as API) and in pharmaceutical dosage forms. The method was validated according to the guidelines of ICH, FDA and USP with respect to accuracy, precision and linearity. For method development a C-18 bonded silica column (250 x 4.6 mm, 5µ, Phenomenex, Inc) was used with a mobile phase comprising of 10% aqueous solution of acetic acid and acetonitrile in a ratio of 80:20 v/v. The flow rate was 0.5 mL/min and effluents were monitored at 300 nm and the retention times were found to be at 7.0±0.1 min and 10.59±0.1 min for levofloxacin and moxifloxacin, respectively. The recovery was found to be more than 99% for each spiked samples of levofloxacin and moxifloxacin, demonstrating the accuracy of the protocol. Intra-day and inter-day precisions of the new method were less than the maximum allowable limit (RSD% ≤ 2.0) according to FDA. The method showed linear response with correlation coefficient value of 0.9975 in both the cases.

Therefore, the developed method was found to be simpler, accurate, reproducible, efficient and less time consuming and can be successfully applied for the simultaneous assay of levofloxacin and moxifloxacin formulations.

Keywords: HPLC, method development, validation, levofloxacin, moxifloxacin.

INTRODUCTION

Levofloxacin, [(-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7oxo-7*H*-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid hemihydrates is the active levo-isomer of racemic ofloxacin (Figure **1**) [1]. It is a synthetic chemotherapeutic antibiotic of the fluoroquinolone class and is used to treat severe bacterial infections or infections not responding to other classes of antimicrobial agents. It possesses wide spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as atypical pathogens such as *Mycoplasma, Chlamydia* and *Legionella* [2]. Levofloxacin also appears to have improved activity against *Streptococcus pneumoniae* compared to ciprofloxacin or ofloxacin [3]. Moxifloxacin, [1-cyclopropyl-6-fluoro-8-methoxy-7-[4aS,7aS)-octahydro-6H-pyrrolo[3,4-b]pyridine-6-yl]-4-oxo-1,4-dihy-droquinoline-3-carboxylic acid is a new generation, 8-methoxyquinolone derivative of fluoroquinolone antibacterial agent (Figure **2**) [4]. Moxifloxacin is active against broad spectrum of pathogens, encompassing Gram-negative, Gram-positive bacteria including *S. pneumoniae* [5, 6]. It is available for oral and parenteral administration.

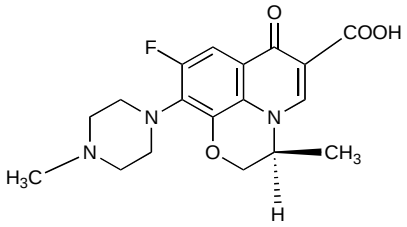

Figure 1: Structure of levofloxacin.

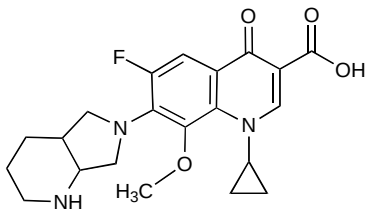

Figure 2: Structure of moxifloxacin.

To ensure the effectiveness of the drug, quality and efficacy assessment and maintenance of proper dosage schedule are of great importance. It has been reported that due to inadequate therapeutic content in antibiotic formulations, susceptible organisms gain

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resistance tremendously and many pathogenic organisms have already shown resistance to a number of antimicrobial drugs [7]. To ensure the desired quality of drugs, manufacturers have to evaluate their products during and after manufacturing processes and at various intervals during the shelf life of the product. Therefore, it is needed to study and determine the potency and efficacy of anti-bacterial preparations like levofloxacin and moxifloxacin, which are commonly prescribed in Bangladesh. Several methods have been previously reported in the literature for determination of levofloxacin and moxifloxacin in the pharmaceutical formulations but there is no report on the simultaneous determination of levofloxacin and moxifloxacin in pharmaceutical dosage forms [8-11]. To the best of our knowledge, this is the first report for the simultaneous determination of levofloxacin and moxifloxacin using non-buffer mobile phase containing 10% aqueous solution of acetic acid with acetonitrile.

Therefore, a rapid and sensitive reversed phase high performance liquid chromatographic method was developed and validated according to the guidelines of FDA, ICH, and USP with respect to accuracy, precision, specificity and linearity [12-14]. The developed method was found to be simpler, accurate, reproducible, efficient and less time consuming, and was applied successfully for the study of levofloxacin and moxifloxacin formulations.

MATERIALS AND METHODS

Working standards of levofloxacin hemihydrates and moxifloxacin hydrochloride were collected from ACI Ltd., Dhaka, Bangladesh with a potency of 95.15% and 98.38%, respectively. For the estimation of levofloxacin and moxifloxacin active raw materials, samples were collected from a renowned pharmaceutical industry of Bangladesh. HPLC grade acetonitrile was procured from local market.

Equipment

HPLC System

High Performance Liquid Chromatographic system (Shimadzu-UFLC Prominence) set with an auto sampler (Model- SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used for the analysis. LC-solutions software was used to record the data.

Column

Analytical reversed phase C-18 ODS column [250×4.60 mm, 5µ, Phenomenex, Inc] was used to analyze the samples.

Mobile Phase

10% aqueous solution of acetic acid and acetonitrile were sonicated for 10 minutes and filtered through a 0.45 µm filter tips. HPLC grade acetonitrile was also filtered and degassed before using.

Chromatographic Conditions

All analyses were done at ambient temperature under isocratic condition. The mobile phase consisted of 10% aqueous solution of acetic acid and acetonitrile in the ratio of 80:20 (v/v) at a flow rate of 0.5 mL/min. The injection volume was 20 µL for standard and samples. Before analysis, every standard and samples were filtered through 0.45 µm filter tips. The column eluate was monitored at 300 nm.

Preparation of Standard Solutions

Accurately weighed 20 mg of levofloxacin was dissolved in 10% acetic acid and made up to 100 mL in a volumetric flask to get a solution having concentration of 200 µg/mL. Similarly 20 mg of moxifloxacin was dissolved in 10% acetic acid and made up to 100 mL in a volumetric flask. Then 50 mL was taken in a 100 mL-volumetric flask from both the solutions of levofloxacin and moxifloxacin, and the solution mixed properly. This contained the concentration of 100 µg/mL. Then by calculation and serial dilution procedure, solutions of various concentrations such as 40, 50, 60, 70, 80 µg/mL were prepared.

Preparation of Test Sample

Ten levofloxacin tablets of a reputed pharmaceutical company were collected from the market, weighed and powdered; and from this powder equivalent to 20 mg of levofloxacin was taken and dissolved in 10% acetic acid in a 100 mL-volumetric flask and the volume was adjusted to 100 mL which results in 200 µg/mL. On the other hand, moxifloxacin hydrochloride is available as ophthalmic solution in market in a concentration of 5 mg/mL of moxifloxacin. 4 mL of this solution was taken and dissolved in 10% acetic acid and made up to 100 mL in a volumetric flask to solution of 200 µg/mL. From each of these solution 50 mL was taken in a 100 mL volumetric flask and mixed properly to get the concentration of 100 µg/mL. 5.5 mL of this solution further diluted to 10 mL with same solvent which results in 55 µg/mL of the mixed drugs.

METHOD VALIDATION

Specificity

The specificity of the method was evaluated to ensure that there was no interference from the

excipients present in the pharmaceutical product. The specificity was studied by injecting the standard solution and pharmaceutical preparation of levofloxacin and moxifloxacin.

Linearity

10% aqueous acetic acid solution was used to dilute standard solution and five different concentration levels (40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL and 80 µg/mL) were prepared. Then 20 µL from each solution was injected into the HPLC by auto-sampler. The analyses were monitored at 300 nm and repeated three times. The average peak areas were plotted against concentrations and calibration curves were used to calculate slope and intercept values as well as to evaluate the linearity of the proposed method by calculating the coefficient of correlation.

Accuracy

The accuracy of an analytical method expresses the closeness between the expected value and the value found. It is expressed by calculating the percent recovery (R%) of the drug recovered. In this case, three successive analyses for three different concentrations of standard solutions of levofloxacin and moxifloxacin (40 µg/mL, 45 µg/mL and 50 µg/mL) were carried out by using the designed method.

Reproducibility

Reproducibility expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample. It was checked by intra- and inter-day repeatability of responses after replicate injections and expressed as %RSD amongst responses using the formula [%RSD = (Standard deviation/Mean) x 100 %]. In the current method development and validation protocol, precision was determined by three replicate analyses of each of the concentrations of 20 µg/mL and 30 µg/mL of standard levofloxacin and moxifloxacin solutions using the proposed method.

RESULTS AND DISCUSSION

A reversed phase HPLC method was developed and validated as per ICH, USP and FDA guidelines for simultaneous determination of levofloxacin and moxifloxacin in pharmaceutical formulations. Both of the drugs were detected at 300 nm using mobile phase comprising of 10% aqueous solution of acetic acid and acetonitrile in the ratio of 80:20 (v/v) at ambient temperature with a flow rate of 0.5 mL/min. The injection volume was kept at 20 µL for standards and samples in both cases. The retention time of levofloxacin and moxifloxacin were found at 7.0±0.1 min and 10.59±0.1 min, respectively (Figure **3**).

The standard solution and market preparations containing levofloxacin and moxifloxacin were analyzed to observe the specificity of the method. No peak was detected close to the retention time of levofloxacin and moxifloxacin which proves the high degree of specificity of the method.

To get the calibration curve, five different concentrations (40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL and 80 µg/mL) were used. When peak areas were plotted against these concentrations, good correlation coefficients (r^2), 0.9975 and 0.998 were obtained for levofloxacin and moxifloxacin, respectively, which were within the acceptable range of guidelines and showed good linear relationship of the newly developed method. The slopes (m) and intercepts (c) of the calibration curve were 146515.3029 and 672467.954 for levofloxacin (Table **1**,

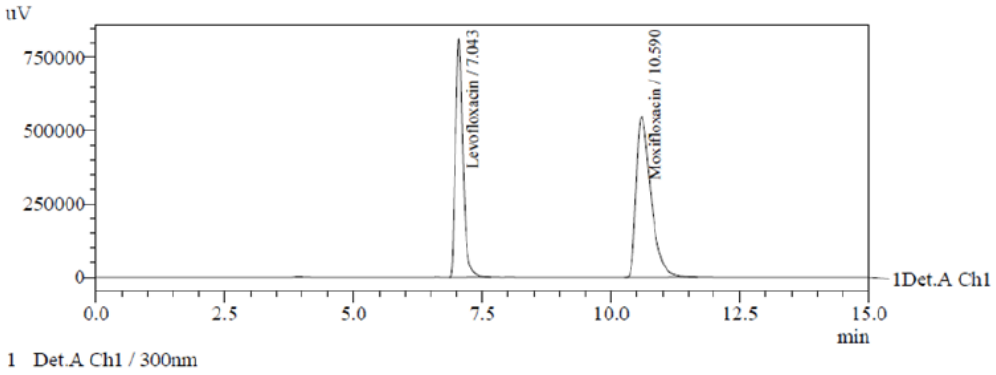

Figure 3: HPLC chromatogram of levofloxacin and moxifloxacin.

Table 1: Linearity of the Method for Levofloxacin

Concentration (µg/mL)	Mean Area (y) (n=3)	Intercept (c)	Slope (m)	Correlation coefficient (r^2)
40	6389854.67	672467.954	146515.3029	0.9975
50	8126350.67			
60	9533757			
70	10976346.33			
80	12290622			

Figure **4**) and 202141.944 and 700570 for moxifloxacin (Table **2**, Figure **5**).

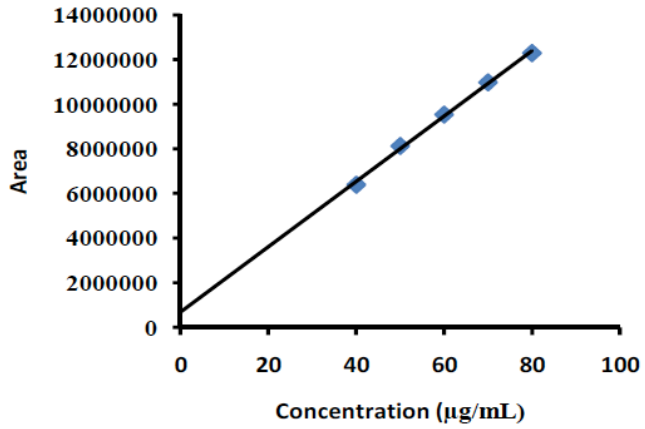

Figure 4: Linearity of curve for standard levofloxacin.

developed method was sensitive enough and accurate for determination of levofloxacin and moxifloxacin.

The reproducibility of the proposed method was checked by intra- and inter-day repeatability of responses after replicate injection of standard solutions (20 and 30 µg/mL). The reproducibility is expressed as %Relative Standard Deviation (%RSD). Levels were analyzed three times within the same day (intra-day variation) and three other days (inter-day variation) (Table **4** and **5**).

Tables (Table **4** and **5**) show the calculations of the %RSD values for intra-day and inter-day, which were found to be <2. These results were within the acceptable range.

Table 2: Linearity of the Method for Moxifloxacin

Concentration (µg/mL)	Mean Area (y) (n=3)	Intercept (c)	Slope (m)	Correlation coefficient (r^2)
40	8645750	700570	202141.944	0.998
50	10898977.33			
60	12878804.67			
70	15039130.33			
80	16682770.67			

The accuracy was evaluated at three different concentrations which were conducted in successive analysis (n = 3) using the proposed method and the values were expressed as percentage of recovery between the mean concentrations found and added concentrations for both of these drugs. For levofloxacin, average percentage of recovery was found to be 98.37%, 104.11% and 99.94% for 40 µg/mL, 45 µg/mL and 50 µg/mL, respectively (Table **3**) and for moxifloxacin average percentage of recovery was found to be 99.55%, 99.71% and 102.32% for 40 µg/mL, 45 µg/mL and 50 µg/mL, respectively (Table **3**). All experimental results were in the range of the acceptability for accuracy [14], which indicated that the

Since, the method was developed and validated according to the guidelines of FDA, ICH and USP with

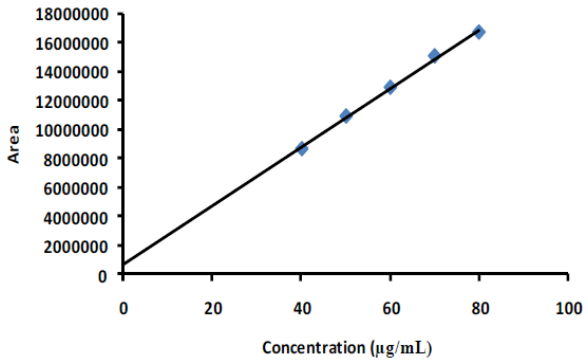

Figure 5: Linearity of curve for standard moxifloxacin.

Table 3: Accuracy of the Developed Method

Injected conc. (µg/mL)	Mean recovered levofloxacin (µg/mL)	% Recovery levofloxacin	Mean recovered moxifloxacin (µg/mL)	% Recovery moxifloxacin
40	39.35	98.37	39.82	99.55
45	46.85	104.11	44.87	99.71
50	49.97	99.94	51.16	102.32

Table 4: Intra-Day Precision of Levofloxacin and Moxifloxacin

Drug	Day	Injected conc. µg/mL)	Mean recovered (µg/mL)	SD	Intra-day %RSD
Levofloxacin	Day 1	20	20.16373	0.344775	0.017099
		30	29.25701	0.1648199	0.563352
	Day 2	20	20.05297	0.038671	0.192844
		30	29.66766	395873	1.334359
	Day3	20	20.72452	0.050881	0.245511
		30	29.04887	0.051675	0.1778898
Moxifloxacin	Day 1	20	19.47814	0.220587	1.132485
		30	29.30477	0.006803	0.023215
	Day 2	20	19.63234	0.240142	1.223196
		30	29.45182	0.003458	0.011741
	Day 3	20	19.86695	0.185884	0.935644
		30	29.21547	0.016092	0.055080

Table 5: Inter-Day Precision of Levofloxacin and Moxifloxacin

Drug	Day	Injected conc. (µg/mL)	Mean recovered (µg/mL)	Mean	SD	Inter-day %RSD
Levofloxacin	Day 1	20	20.16373	20.31374	0.360031	1.772352
	Day 2		20.05297			
	Day 3		20.72452			
	Day 1	30	29.25701	29.32451	0.314869	1.073740
	Day 2		29.66766			
	Day 3		29.04887			
Moxifloxacin	Day 1	20	19.47814	19.65914	0.195786	0.995903
	Day 2		19.63234			
	Day 3		19.86695			
	Day 1	30	29.30477	29.32402	0.119345	0.406987
	Day 2		29.45182			
	Day3		29.21547			

respect to accuracy, precision and linearity, it may be suitable for routine analysis of samples and APIs. %Recovery for samples were also calculated which were within the expected limit (Table **6**) [12-15].

Table 6: Determination of Drug Content Found in the Levofloxacin and Moxifloxacin Formulations

Drug	Sample code	Injected conc. (µg/mL)	Mean peak area	Intercept (c)	Slope (m)	Recovered conc. (µg/mL)	% Recovery
Levofloxacin	Sample -1	55	8775922	672467.95	146515.30	55.31	100.56
	Sample-2	55	8690673			54.73	99.50
	Sample-3	55	8862801			55.90	101.64
Moxifloxacin	Sample-1	55	12112392	700570	202141.94	56.46	102.64
	Sample-2	55	12143002			56.61	102.92
	Sample-3	55	12126139			56.521	102.77

CONCLUSION

The efficacy of a drug depends on some absolute requirements such as quality, potency etc. It is obvious that a little change in the formulation or variations in the manufacturing process or use of low quality materials including APIs can affect the efficacy of the drugs leading to harmful effects to the patients. Therefore, quality and efficacy assessment and maintenance of proper dosage schedule are strongly needed to ensure the effectiveness of the drug. Hence, we planned to study the potency of most commonly used antibacterial preparations like levofloxacin and moxifloxacin which are frequently prescribed in Bangladesh.

To attain this objective, a rapid and sensitive reversed phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated according to the guidelines of FDA, ICH and USP with respect to accuracy, precision, specificity and linearity. The newly developed method has been found to be simpler, accurate, reproducible, efficient and less time consuming, and has been applied successfully for the simultaneous study of levofloxacin and moxifloxacin.

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