International Journal of Pharmacy Research and Technology
2013, Volume 3, Issue 2, 17-21
ISSN 2250 – 0944 (Online)
ISSN 2250 – 1150 (Print)

Research Article

Simultaneous Separation of Five Fluoroquinolones - Norfloxacin, Moxifloxacin, Enrofloxacin, Sparfloxacin and Prulifloxacin Using an Isocratic HPLC: Application to Determination of Norfloxacin in Pharmaceutical Formulations

Ravisankar Panchumarthy[*a,b]
*[a]Faculty of Science, Sri Chandrasekharendra Saraswathi Viswa Maha Vidyalaya (SCSVMV University), Enathur, Kanchipuram – 631561 (T.N.) India.
[b]Department of Pharmaceutical Analysis and quality Assurance, Vignan Pharmacy College, Vadlamudi, Guntur – 522213
***Corresponding Author** E-mail: banuman35@gmail.com Mobile: 09000199106
Received: 12/06/2013, Revised: 05/10/2013 Accepted: 20/10/2013

ABSTRACT

A rapid, simple, precise, specific, highly sensitive, accurate and reproducible isocratic reversed phase HPLC method was developed for the separation of five fluoroquinolones and validated for determination of norfloxacin for routine pharmaceutical quality control analysis in bulk and formulations. One of the salient aims for the method development is to accomplish constant, reproducible separation. The selection of good method is essential if this goal is to be achieved. RP-HPLC method was developed by using WELCHROM C_{18} Column (4.6 X 250mm, 5µm), SHIMADZU LC-20AT prominence liquid chromatograph. The mobile phase consisting of phosphate buffer (pH-6.8)and acetonitrile in the proportion of 50:50 v/v under isocratic elution at a flow rate of 1mL/min was employed. The responses were measured at 281nm using SHIMADZU SPD-20A prominence UV-Vis detector. The retention time of norfloxacin, moxifloxacin, enrofloxacin, sparfloxacin and prulifloxacin were found to be 2.940 min, 3.363 min, 3.790 min, 4.013 min and 7.520 min, respectively. Chromatographic runtime was 10 min with elution window of 6 min and with a resolution of greater than 2.0 for all compounds. The developed method was validated according to ICH guidelines.The average recoveries ranged from 99.03 - 100.36 %, with %RSD less than 2%. The concentration of calibration solutions was in the range of 2-10 µg/mL and LOD and LOQ were 0.1491 and 0.4519 µg/mL respectively. The method was successfully applied to analysis of norfloxacin in pharmaceutical formulation. It can also be extended for the determination of other four fluoroquinolones or their combinations.

Keywords: Norfloxacin, moxifloxacin, enrofloxacin, sparfloxacin, prulifloxacin, RP-HPLC.

INTRODUCTION

The fluoroquinolones are a family of synthetic broad-spectrum antibacterial drugs[1]. The majority of quinolones in clinical use have a fluorine atom attached to the central ring system, typically at the C_6 or C_7 position. Quinolones obstruct the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation. For many Gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria.These five fluoroquinolones are broad spectrum antimicrobials with potent activity against Gram+ve and Gram –ve bacteria.

Norfloxacin is 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline-3-carboxylic acid, a second-generation synthetic antibacterial agent of the fluoroquinolone class.It is indicated for the treatment of a wide variety of infections caused by susceptible gram-positive & gram-negative organisms including mixed infections caused by two or more organisms. Norfloxacin is used in treatment of Urinary Tract Infections e.g. acute & chronic pyelonephritis, prostatitis, cystitis, epididymitis& chronic complicated or recurrent UTIs, Gonorrhea, Gastrointestinal Tract Infections e.g. enteric fever, bacterial diarrheas, intra-abdominal Infections e.g.peritonitis, intra-abdominal abscess, cholecystitis, prophylaxis of sepsis in neutropenic patients: from the bacterial flora, severe systemic Infections e.g. septicemia, bacteremia & infections in immuno-compromised patients.

Literature survey revealed that very few methods have been reported for the analysis of various fluoroquinolones which include spectrophotometry [2-4], RP-HPLC [5-11]. Several HPLC methods had been developed for determination of these drugs individually or in combination with other drugs but no HPLC method for simultaneous estimation of these five drugs using C_{18} column with isocratic conditions has been reported till date. Several HPLC methods had been developed for determination of these drugs individually or in combination with other drugs but no HPLC method for simultaneous estimation of these six drugs using C_{18} column with isocratic conditions has been reported till date. It can also be applied for routine analysis of either alone or of any combinations of the above mentioned drugs in dosage forms.

MATERIALS AND METHODS

Instruments and Chromatographic conditions

Chromatographic separations were attained by using Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was injected into the HPLC system. The HPLC system was equipped with “Spinchrom” data acquisition software. Separations were performed on the reversed phase column using a mobile phase consisting of phosphate

buffer (pH-6.8) and acetonitrile in the proportion of 50:50 v/v. The mobile phase was delivered at a flow rate of 1 mL/min. Eluate was monitored at 281 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

Standards and chemicals used:

Fluoroquinolone samples of Norfloxacin (NOR) was provided by Cipla Ltd., Prulifloxacin (PRF) by Hetero Labs, Sparfloxacin (SPA) by Ananth Pharmaceuticals, Moxifloxacin (MOX) by Torrent Pharmaceuticals and Enrofloxacin (ENF) from Sri Valli organics (Figure 1). All chemicals were analytical grade. KH_2PO_4 and $K_2HPO_4$are purchased from S.D Fine-Chem. Ltd., Mumbai, India. Acetonitrile (HPLC grade) is obtained from Merck Pharmaceuticals Private Limited, Mumbai, India. Commercial tablets of NOR were purchased from local market. NORILET tablets containing 400mg of NOR are manufactured by Dr.Reddy's Lab Ltd., Hyderabad, India.

Table 1 Optimized chromatographic conditions and system suitability parameters of proposed RP-HPLC method for Norfloxacin

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Phosphate buffer (pH-6.8): Acetonitrile = 50:50, v/v.
Mobile phase	Phosphate buffer (pH-6.8): Acetonitrile = 50:50, v/v.
Flow rate	1 mL/min.
Detection wave length	UV at 281 nm.
Run time	10 minutes
Column back pressure	118 - 119 kgf
Temperature	Ambient temperature(25°C)
Volume of injection loop	20 µL
Retention time (t_R)	2.953 min
Theoretical plates [th.pl] (Efficiency)	9861
Theoretical plates per meter [t.p/m]	197228
Tailing factor (asymmetry factor)	1.062

Preparation of mobile phase

1.488g of KH_2PO_4 and 0.288g of K_2HPO_4 were accurately weighed and dissolved in 500 mL of HPLC grade water. The above prepared buffer and acetonitrile were mixed in the proportion of 50:50 v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of calibration standards

About 100 mg of pure NOR was accurately weighed and dissolved in 100 mL of mobile phase to get 1 mg/mL stock solution. Working standard solution of NOR was prepared with mobile phase. To a series of 10mL volumetric flasks, standard solutions of NOR in the concentration range of 2, 4, 6, 8, 10µg/mL were transferred. The final volume was made with the mobile phase and similarly 10µg/mL of each other standard fluoroquinolones were prepared from 1 mg/mL stock solutions of MOX, ENF, PRF andSPA respectively into each10mL volumetric flask.

Calibration curve of Norfloxacin

Replicates of each calibration standard solutions 2, 4, 6, 8 and 10 µg/mL were injected into the chromatograph, the retention times and average peak areas were recorded. Calibration graph was plotted by taking concentration of NOR on X-axis and peak areas of standard NOR on Y-axis and the regression equation data is computed.

Assay of Marketed Formulations of Norfloxacin:

The content of twenty tablets was transferred into a mortar and ground to a fine powder. From this tablet powder a quantity equivalent to 100 mg of NOR was taken and the drug was extracted in 100 ml of mobile phase. The resulting solution was filtered through 0.25 µm nylon membrane filter and degassed by sonication. This solution was further suitably diluted for chromatography. The test solutions were injected into the system by filling a 20 µL fixed volume loop manual injector. The elutes were monitored with UV detector at 281 nm. The amount of drug present in sample was computed from the calibration graph

.

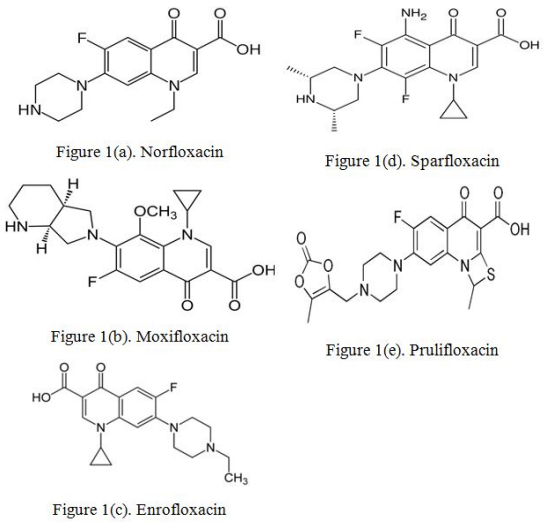

Figure 1(a). Norfloxacin

Figure 1(d). Sparfloxacin

Figure 1(b). Moxifloxacin

Figure 1(e). Prulifloxacin

Figure 1(c). Enrofloxacin

Figure 1 Structures of fluoroquinolones investigated in thepresent study

VALIDATION OF QUANTITATIVE HPLC METHOD

The developed method of analysis was validated as per the ICH [12] for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

System suitability: Set up the chromatographic system, allow the HPLC system to stabilize for 40 min. Inject blank

preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters.

Specificity: The specificity of the proposed method was established by reviewing the effect of various excipients and other additives usually present in the preparations of NOR in the determinations under ideal conditions. The blank, standard, placebo, placebo spiked with analyte and test preparations were analyzed as per the method to examine the interference of blank and placebo with NOR peaks.

Linearity: Linearity for NOR was determined by fixing standard solutions at different concentrations from 50% to 150% of the test concentration. The linearity graphs for the suggested assay methods were acquired over the concentration range of 2-10 µg/mL of NOR. A calibration curve was designed between concentration and peak area response and statistical analysis of the calibration curve was accomplished.

Table 2 Chromatogram results of proposed combination of Fluoroquinolones

Sample	Retention time, (t_R), min.	Assymmetry	Efficiency (theoretical plates)	Resolution
Norfloxacin	2.940	1.078	5497	—
Moxifloxacin	3.363	1.067	5869	2.540
Enrofloxacin	3.790	1.020	13539	2.797
Sparfloxacin	4.013	1.069	9549	2.040
Prulifloxacin	7.520	1.001	15984	17.484

Precision: Intra-day and inter-day precision of the procedure were determined by performing six determinations at the same concentration (10µg/mL) of NOR during the same day, under the same experimental conditions and on a different day respectively. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

Accuracy/Recovery: The accuracy of the method was assessed in triplicate at 3 different concentrations equivalent to 50%, 100% and 150% of the active ingredient, by adding a known amount of NOR standard to a sample with pre-determined amount of NOR. The recovered amount of NOR, % RSD of recovery, % recovery of each concentration is calculated to determine the accuracy.

Table 3: Assay results of Norfloxacin formulation

Formulations	Labeled amount	Amount found*	% Assay±SD*
NORILET tablets (Dr.Reddy's Lab Ltd., India)	400 mg/tablet	396.202 mg/tablet	99.050 ± 1.011 %

*****Average of 6 determinations; SD is standard deviation.

Robustness: The Robustness of developed analytical method was proven by the analysis of NOR under different experimental conditions such as making deliberate changes in chromatographic conditions like flow rate (± 0.2 ml/min), detection wavelength (±5 nm) and Mobile phase composition (±5%).

LOD and LOQ: Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve.

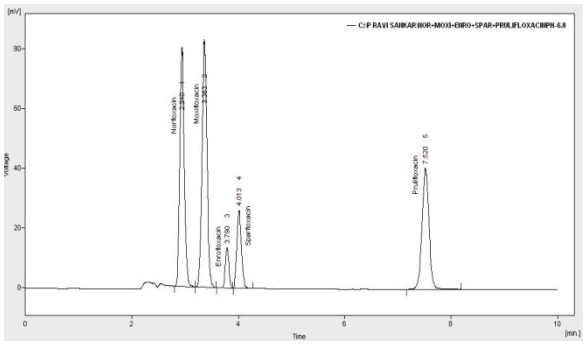

Figure 2 Chromatogram of mixture of five Fluoroquinolones

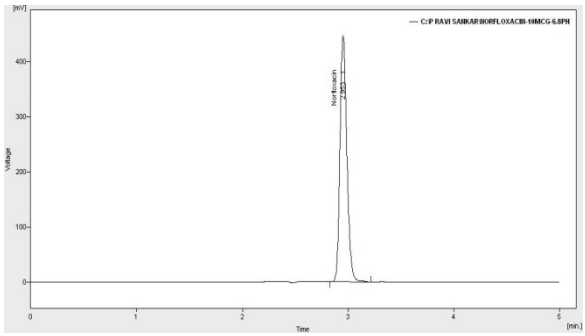

Figure 3 Standard Chromatogram of Norfloxacin (10µg/ml)

RESULTS AND DISCUSSION

The present study was aimed at develop a rapid, accurate and precise HPLC method for the separation of five fluoroquinolones and subsequent determination of NOR in pharmaceutical dosage forms. In order to set up analysis of the component peaks under isocratic conditions, mixtures of phosphate buffer, acetonitrile in different combinations were tested as mobile phase on a C_{18} stationary phase. A combination of phosphate buffer (pH – 6.8) and acetonitrile in a ratio of 50:50, v/v, was used as a mobile phase at a flow rate of 1mL/min was proved to be the most suitable of all combinations of mobile phase tried since the chromatographic peak obtained was well shape symmetrical peak. The wave length of detection was set at 281 nm from UV overlain spectra of drugs under study. The system suitability was carried out on freshly prepared NOR standard solution for the evaluation of system suitability parameters such as retention time, peak area, peak tailing and number of theoretical plates, LOD and LOQ. Six replicate injections for system suitability test were injected into the chromatographic system and system suitability results are given in Table 1. The specificity results were found that there was no interference due to excipients in the

tablet formulation and also that there is good correlation between the retention times of the standard and sample. Under the conditions described above separation of the fluoroquinolone mixture was achieved with a run time of 10 min, with an elution window of 6 min for all five analytes. The retention times for NOR, MOX, ENF, SPA and PRF in mixture were found to be2.940 min, 3.363 min, 3.790 min, 4.013 min and 7.520 min, respectively. The separation chromatogram of mixture is shown in Figure2.The retention times and peak areas of five fluoroquinolones in combination were shown in Table 2.The calibration curve obtained by concentration on X-axis and peak area on Y-axis shows the linearity in the concentration range of 2-10 µg/mL of NOR and the linearity graph is shown in Figure 3. The regression equation was found to be Y=283.21X-8.6932 with regression coefficient of R^2 = 0.9998 which indicates this method had good linearity. The developed method was applied to the assay of NOR tablets. The results were very close to labeled value of commercial tablets. The representative standard chromatogram of NOR is shown in Figure 4 and the assay results are presented in Table 3. Precision was studied to find out intra-day and inter-day variations in the test methods of NOR for three times on the same day and on different days. The %RSD for intra-day and inter-day precision variations studied at 10µg/mL obtained was 0.4176 and 0.5717 respectively which is within the acceptable criteria of NMT 2.0. This reveals that the proposed method is quite precise. The % recoveries of the drug solutions were studied at 3 different concentration levels. The % individual recovery and the % RSD values at each level were within the acceptance limits. Generally the mean percentage recovery of NOR at each level was not less than 99% and not more than 101%. Satisfactory recoveries ranging from 99.03 - 100.36% were obtained by the proposed method. Robustness was done by deliberate changes in the chromatographic conditions like mobile phase flow rate, temperature, mobile phase composition etc. Deliberate changes in developed method had not much affected the peak tailing, theoretical peaks and % assay which demonstrated that the developed method was Robust in nature. A summary of validation parameters for the proposed method is presented in Table 4.

Table 4: Summary of Validation parameters for the proposed method for Norfloxacin:

Parameter	Method
Linearity range (µg/mL)	2-10 µg/ml
Regression equation (Y = a+bx)	Y= -8.6932 + 283.21X
Slope(b)	283.21
Intercept(a)	-8.6932
Correlation coefficient (r)	0.9999
Regression coefficient (R^2)	0.9998
% Relative standard deviation* i.e., Coefficient of variation (CV)	0.9850
Accuracy (% of Recovery)	99.03 – 100.36 %
Precision	
Intra-day S.D and % RSD*	0.0413 and 0.4176
Inter-day S.D and % RSD*	0.0562 and 0.5717
Limit of detection (µg/mL)	0.1491µg/mL.
Limit of quantitation(µg/mL)	0.4519µg/mL.
Percentage range of errors*(Confidence limits)	
0.005significance level	0.8679
0.001 significance level	1.3614

*Average of 6 determinations; Acceptance criteria < 2.0.

CONCLUSION

A new validated RP-HPLC method has been developed for the determination of NOR and Rapid separation of selected five fluoroquinolones with a relatively short retention time, provides phenomenal resolution, excellent peak shape, gave consistent and highly reproducible results on C_{18} HPLC column. The method overall proved to be economical, simple, rapid, precise, very sensitive, cost-effective, time saving, robust and accurate. It can be reliably used for determination of the said five fluoroquinolones in short periodand even in small concentrations. By using this method one can elute all the five drugs within ten minutes. This method was completely validated shows excellent results and also free from interference of the other additives used in the formulations. Under the conditions described above, separation of the five fluoroquinolone agent mixture was achieved with a total run time of 10 minutes with an elution window of 6 minutes for all five analytes. The ease in preparation of mobile phase and economy of the components of mobile phase make this method the best choice in routine analysis of NOR, MOX, ENF, SPA and PRF in bulk and their pharmaceutical formulations. This method provides decorous resolution power with short runtimes, good retention, superior peak shape and excellent reproducibility of the results achieved. Therefore it is suitable for routine analysis of above said anti-bacterial agents. So it could be used for rapid and reliable determination of the above described anti-bacterial fluoroquinolone agents, irrespective of their concentration levels or in combinations.

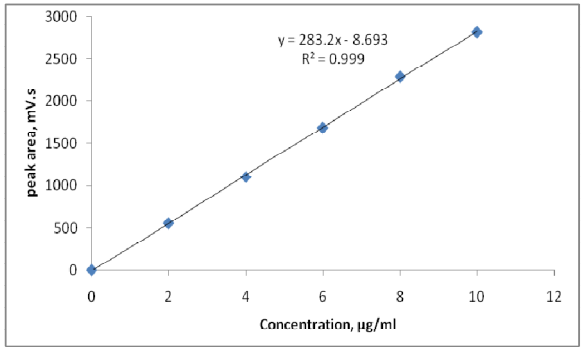

Figure 4 Calibration Plot of Norfloxacin

ACKNWOLEDGEMENT

The authors would like to acknowledge Hetero Labs for providing the sample of Prulifloxacin. They also thank Cipla Ltd., for proving the gift sample of Norfloxacin. We whole heartedly thank Ananth Pharmaceuticals, Torrent pharmaceuticals for their samples drugs Sparfloxacin and Moxifloxacin. We are thankful to Sri Valli organics for providing Enrofloxacin sample. We are highly grateful to Dr. L. Rathaiah, Honorable Chairman, Vignan group of institutions, and principal of Vignan pharmacy college, Dr. P.Srinivasa Babu for providing the necessary facilities to carry out this research work.

REFERENCES

1. Drug today medical journal, Lorina publication (India) Inc., Delhi-91, DT 78, 1:512-554 (2012).
2. P. Ravisankar, Ch. Devadasu, P. Srinivasa Babu, G. Devala Rao, S. Gananathamu, S. Sowjanya, New spectrophotometric methods for the Quantitative

analysis of prulifloxacin in Pharmaceutical dosage forms, Int. J. Chem. Sci., 8(4):2309-2324 (2010).
3. Noura H, Abou-Taleb, Dina T. El-Sherbiny, Dalia R. El-Wasseef, Mohamed A. Abu El-Enin, Saadia M. El-Ashry, Simultaneous Determination of Norfloxacin and Tinidazole Binary Mixture by Difference Spectroscopy, Int. J. Biomedical Sci., 7(2):137-144 (2011).
4. Mahesh Attimarad, Bander E Al-Dhubiab, Ibrahim A Alhaider, et al., Simultaneous determination of moxifloxacin and cefixime by first and ratio first derivative ultraviolet spectrophotometry, Chemistry Central J., 6:105 (2012).
5. Samanidou VF, Demetriou CE, Papadoyannis IN, Direct determination of four fluoroquinolones, enoxacin, norfloxacin, ofloxacin, and ciprofloxacin, in pharmaceuticals and blood serum by HPLC, Analytical bioanalytical Chem., 375(5):623-629 (2003).
6. Ravisankar P, Devala Rao G, Gopala Reddy P, Rapid simultaneous separation of fluoroquinolone antibacterial - levofloxacin, sparfloxacin and balofloxacin by isocratic RP-HPLC: application to sparfloxacin determination in pharmaceutical dosage forms. J. Chem. Pharm. Sci., 6(2):120-133 (2013).
7. Sun H., Qiao F, Liu G, Liang S, Simultaneous isolation of six fluoroquinolones in serum samples by selective molecularly imprinted matrix solid-phase dispersion, Analytica Chimica Acta, 625(2):154-159 (2008).
8. Du L., Wei H, Zhang J, Zhang Q, Separation and Determination of Six Fluoroquinolones by Reversed-Phase High Performance liquid Chromatography, Chinase J. Chromat. 21(1) 503-506 (2003).
9. Syed Naeem Razzaq, Islam Ullah Khan, Irfana Mariam, Syed Saleem Razzaq, Stability indicating HPLC method for the simultaneous determination of moxifloxacin and prednisolone in pharmaceutical formulations, Chem. Central J., 6:94 (2012).
10. P. Ravisankar, G. Devala Rao, Ch. Devadasu, G. Sudhakar Saibabu, P. Srinivasa Babu, A validated RP-HPLC method for the assay of Prulifloxacin in marketed drug product using Levofloxacin as an internal standard, Int. J. Che. Sci., 11(1):95-105 (2013).
11. Espinosa-Mansilla, A, Peña A, Gómez DG, Salinas F, HPLC determination of enoxacin, ciprofloxacin, norfloxacin and ofloxacin with photoinduced fluorimetric (PIF) detection and multiemission scanning: Application to urine and serum, J. Chromatography B, 822(1):185-193 (2005).
12. ICH, Q2B, Harmonized Tripartite Guideline, Validation of Analytical Procedure: Methodology, IFPMA, in: Proceedings of the International Conference on Harmonization, Geneva, March 1996.