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RAPID SIMULTANEOUS SEPARATION OF FLUOROQUINOLONE ANTIBACTERIAL - LEVOFLOXACIN, SPARFLOXACIN AND BALOFLOXACIN BY ISOCRATIC RP-HPLC: APPLICATION TO SPARFLOXACIN DETERMINATION IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A selective, sensitive, rapid, economical, reproducible and accurate isocratic reversed phase high performance liquid chromatographic method was developed with UV detection and was validated and applied for the determination of fluoroquinolones in pharmaceutical dosage forms. The effects of mobile phase composition, buffers, pH, and acetonitrile concentrations were investigated on the separation of three fluoroquinolones – levofloxacin, sparfloxacin and balofloxacin were assayed without any interference. 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 used was phosphate buffer (pH-3.1): acetonitrile (70:30 v/v) with a flow rate of 1ml/min. The responses were measured at 293nm using SHIMADZU SPD-20A prominence UV-Vis detector. The recovery, selectivity, linearity, precision and accuracy of the method were evaluated from spiked tablet samples according to ICH guidelines. The method was successfully applied to sparfloxacin pharmaceutical dosage form. It can also be extended for the determination of other two fluoroquinolones.

KEY WORDS: Isocratic, RP-HPLC, levofloxacin, sparfloxacin, balofloxacin, method Validation.

INTRODUCTION

Fluoroquinolones (John S Wolfson and David C Hooper, 1985) are a class of compounds that comprise a large and expanding group of synthetic antimicrobial agents. Structurally (Lance R Peterson, 2001), all fluoroquinolones contain a fluorine atom at the 6-position of the basic quinolone nucleus. Quinolonesact by inhibiting the activities of DNA gyrase (L.Mark Fisher, 1989) (enzyme catalyzing changes in the degree of double stranded DNA super coiling) in gram-negative bacteria, which in turn inhibit replication and transcription of bacterial DNA. Quinolones similarly inhibit the *in vitro* activities of DNA topoisomerase IV (Katie, 2013) enzyme mediating relaxation of duplex DNA and the unlinking of daughter chromosomes following replication which is believed to be the primary target in gram-positive bacteria. These three fluoroquinolones are broad spectrum antimicrobials (Furet and Pechere, 1990) with potent activity against Gram+ve and Gram –ve bacteria. The new fluoroquinolone sparfloxacin (5-Amino-1-cyclopropyl-7-(*cis*-3,5-dimethyl-1-piperazinyl)-6,8-difluoro-1,4dihydro-4-oxo-3-quinolinecarboxylic acid), is a broad spectrum fluorinated a quinolone antibiotic, prescribed for infective ophthalmitis and sinusitis, acute exacerbation of chronic bronchitis, community-acquired pneumonia, eye infections, urinary tract infection.

Sparfloxacin is a new difluorinated quinolone with similar activity for gram-negative and gram-positive bacteria and a spectrum of activity that includes anaerobes, Chlamydia trachomatis, Mycoplasma spp., and mycobacteria (Laura and Zhu, 1991). The quinolones and sparfloxacin compounds are bactericidal in nature. The molecular target of quinolones is considered to be DNA gyrase, since quinolones inhibit gyrase activities and gyrase isolated from quinolone-resistant strains are resistant to quinolones (Gellert, 1977) (Sato, 1986) (Sugino, 1977). Escherichia coli gyrase (EC 5.99.1.3) consists of subunits A and B, which are the products of the gyr A and gyr B genes, respectively (Higgins, 1978) (Mizuuchi, 1978). Since the unexpected finding by Shen and Pernet that [3H] norfloxacin binds to DNA but not to purified gyrase (Shen, 1985), it has been proposed that quinolones may inhibit gyrase activity through binding to DNA. This proposal still exists after the later finding that a new quinolone binding site appears upon the formation of gyrase-DNA complexes and that quinolone binding to the site is closely correlated with inhibition of gyrase activity (Shen, 1989).

Literature survey revealed that very few methods have been reported for the analysis of sparfloxacin which include luminescence spectroscopy, Reverse Phase High Pressure liquid Chromatography, LC-MS, HPLC with fluorescent spectroscopy, RP-HPLC with fluorescence detection, HPLC-Electro spray ionization mass spectroscopy[7] and few UVspectrophotometric methods[8] The present study illustrates development and validation of simple, sensitive, precise and accurate RP-HPLC method for the determination of new antibacterial fluoroquinolone sparfloxacin in bulk samples and pharmaceutical tablet dosage forms as per ICH guideline.

The goal of this study is to develop rapid HPLC methods for the analysis of sparfloxacin in bulk drug samples and tablet formulations using the most commonly employed column (C_{18}) with UV detection at appropriate wavelength. 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 three drugs using C_{18} column with isocratic conditions has been reported till date.

In the present proposed work a successful attempt had been made to develop a method for the simultaneous separation of sparfloxacin (Ming Li, 2000) (Borner E, 1992) (Kamberi, 1999) (Lyon, 1994) (Dorota, 2011) (Mohmoud, 2011) (Somia Gul, 2012) (Himanshu, 2010) (Gupta, 2010) (Marona, 1999), levofloxacin (kalta, 2008) (Vipul, 2009) (Shervington, 2005) (Srinivas, 2008) (Sun, 2012) (Yu, 2012) and balofloxacin (Merck, 2006) (Sean, 2011) (Marutani, 1993) (Bain, 2007) (Kozawa, 1996) (Uematsu, 1994) (Bain, 2007) (Nakagawa, 1995) and the successful attempt had been made to estimate third generation fluoroquinolone sparfloxacin tablet dosage form and validate it. This method can also be extended for the determination of other two said fluoroquinolones. From the economical point of view and for the purpose of routine analysis, it was decided to develop a more economical RP_HPLC method with simple mobile phase preparation for the estimation of above said drugs. The method would help in assay of drugs in single run which reduces the time of analysis and does not require separate method for each drug. Thus, this paper reports an economical, simple and accurate RP_HPLC method for the determination of above said pharmaceutical dosage forms.

2. MATERIALS AND METHODS

Quantitative HPLC was performed on a high pressure isocratic high performance liquid chromatograph (SHIMADZU LC-20AT prominence liquid chromatograph) with two LC-20AT VP pumps, manual injector with loop volume of 20 µl (Rheodyne), programmable variable wavelength SHIMADZU SPD-20A prominence UV-Vis detector and WELCHROM C_{18} Column (4.6 X 250mm, 5µm particle size). The HPLC system was equipped with "Spinchrom CFR" software. In addition an electronic balance (shimadhu 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: Sparfloxacin pharmaceutical grade was kindly supplied as gift sample by Ananth Pharmaceuticals. Quinolone samples of BLFX & LEV were provided by Hetero Labs and Aristo Pharma respectively. All chemicals were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from S.D Fine-Chem. Ltd., Mumbai, India. While acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Limited (Mumbai, India). Commercial tablets of sparfloxacin was procured from local market and used for analysis of marketed formulation. Sparcip100mg (cipla), spardac 200mg (lupin) Ltd., Mumbai, India.

Preparation of mobile phase: A 10 mM phosphate buffer was prepared by dissolving 6.056 g of potassium dihydrogen orthophosphate in 445 ml of HPLC grade water. To this 55ml of 0.1M phosphoric acid was added and pH was adjusted to 3.1 with triethylamine. The above prepared buffer and acetonitrile were mixed in the proportion of 70: 30 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 SPFX was accurately weighed and dissolved in 100 ml of mobile phase to get 1 mg/ml stock solution. Working standard solution of sparfloxacin was prepared with mobile phase. To a series of 10ml volumetric flasks, standard solutions of sparfloxacin in the concentrations range of 2, 4, 6, 8, 10 µg/ml were transferred. The final volume was made with the mobile phase and similarly 10 µg/ml of each other standard fluoroquinolones was prepared from 1 mg/ml stock solutions of LEV, BLFX respectively into each10ml volumetric flask.

System suitability: System suitability tests are an integral part of chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10 µg/ml for SPFX to check the reproducibility of the system.

At first the HPLC system was stabilized for forty min. One blank followed by six replicates of a single calibration standard solution of sparfloxacin was injected to check the system suitability. To ascertain the systems suitability for the proposed method, a number of parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken and results were presented in Table 1.

Recommended procedure:

Calibration curve for sparfloxacin: Replicates of each calibration standard solutions (2,4,6,8,10 µg/ml) were injected using 20µl fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting by taking concentrations of SPFX on X-axis and ratio of peak areas of standard sparfloxacin on Y-axis and regression equations were computed for SPFX (Table 2).

Analysis of marketed formulation: The content of twenty tablets was accurately weighed and transferred into a mortar and ground to a fine powder. From this, tablet powder which is equivalent to 100 mg of SPFX was taken and the drug was extracted in 100 ml of mobile phase. The resulting solution was filtered through Whatman Grad No 1 filter paper 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 chromatographic run time of 10 min. was maintained for the elution of the drug from the column. The elutes were monitored with UV detector at 293 nm. A 20µlvolume of standard and sample solutions were separately injected on HPLC system. From the peak area of SPFX the amount of drug in the sample were computed. The content was calculated as an average of six determinations and experimental results were presented in table 3.The representative standard and sample chromatograms of SPFX were shown in fig.6 and fig. 7.

Validation study of sparfloxacin: An integral part of analytical method development is validation. Once the method has been developed, it is necessary to evaluate under the expected conditions for real samples before being used for the specific purpose. The proposed method of analysis was validated as per the ICH [39] and USP [40] guidelines for the parameters including specificity, Precision, accuracy, linearity, robustness, system suitability, limit of detection (LOD) and limit of quantification (LOQ).

Specificity: The effect of wide range of excipients and other additives usually present in the formulations of SPFX in the determinations under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been added to the sample solution injected and tested.

Precision: Intraday and Interday precision study of SPFX is carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 10µg/ml. The percent relative standard deviation (% RSD) is calculated which is within the acceptable criteria of not more than 2.0.

Linearity: The linearity graphs for the proposed assay methods were obtained over the concentration range of 2,4,6,8,10 µg/ml SPFX. Method of least square analysis is carried out for getting the slope, intercept and correlation coefficient, regression data values and the results were presented in Table 2. The representative chromatograms indicating the SPFX were shown in Fig. 8 to 12. A calibration curve was plotted between concentration and area response and statistical analysis of the calibration curve was shown in Fig. 15.

Accuracy (Recovery studies): The accuracy of the method is determined by calculating recovery of sparfloxacin by the method of addition. Known amount of sparfloxacin at 50%, 100%, and 150% is added to a pre quantified sample solution. The recovery studies are carried out in the tablet in triplicate each in the presence of placebo. The mean percentage recovery of sparfloxacin at each level is not less than 99% and not more than 101%.

Robustness: The Robustness is evaluated by the analysis of sparfloxacin under different experimental conditions such as making small changes in flow rate (± 0.2 ml/min), λmax (± 5), Mobile phase composition (±5%), and pH of the buffer solution.

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=3.3(SD)/S and LOQ=10(SD)/S, where SD= standard deviation of response (peak area) and S= average of the slop of the calibration curve.

3. RESULTS AND DISCUSSION

Reversed phase HPLC method was preferred for the determination of LEV, SPA and BLFX. Preliminary experiments were carried out to achieve the best chromatographic conditions for the simultaneous determination of

the drug substances. Several column types and lengths were tried considering other chromatographic parameters. C_{18} column with a 4.6 mm inner diameter and 5µm particle size was chosen. Spectroscopic analysis of drugs showed that levofloxacin, sparfloxacin, balofloxacin have maximum UV absorbance at λmax 293, 291, 293 respectively. UV overlain spectra of these drugs showed that these drugs absorbed appreciably at 293 nm, so that this wavelength was selected as the detection wave length. Chromatographic conditions were optimized by changing the mobile phase composition & buffers used in the mobile phase. Different experiments were performed to optimize the mobile phase but adequate separation of drugs could not be achieved. By altering the pH of buffer from 4.5 to 3.1 a good separation was achieved. Different proportions of solvents were tested. Eventually the best separation was obtained by the isocratic elution system using a mixture of phosphate buffer (pH-3.1): acetonitrile (70:30, v/v) pH of buffer adjusted to 3.1 using orthophosphoric acid at a flow rate of 1 ml/min. A typical chromatogram for simultaneous estimation of the three drugs obtained by using a forementioned mobile phase. Under these conditions levofloxacin, sparfloxacin, and balofloxacin were eluted at 3.613min, 5.500min and 6.257minutes respectively with a run time of 10 minutes. The representative chromatogram of this simultaneous estimation shown in fig no 2 and results are summarized in table no 9.For the comparative evaluation of retention times & peak areas of fluoroquinolones, chromatograms of these three quinolone standards recorded individually. The representative individual standard chromatograms of the three fluoroquinolones are shown in fig no 3 to 5 and the results were presented in table no 10.

The mobile phase consisting of phosphate buffer (PH-3.1): acetonitrile (70:30% v/v) at1ml/min flow rate was optimized which gave sharp peak, minimum tailing factor with short runtime for SPFX. The retention time for SPFX was 5.497 min. UV spectra of SPFX showed that the drug absorbed maximum at 293 nm, hence this wavelength was selected as the detection wavelength. System suitability parameters & optimized chromatographic conditions are shown in Table no 1. The calibration curve for SPFX was found to be linear over the range of 2,4,6,8,10µg/ml. The data of regression analysis of the calibration curve is shown in Table 2.The developed method was applied to the assay of SPFX tablets. The experimental results are given in Table 3. The results were very close to labeled value of commercial tablets. The representative standard and sample chromatograms of SPFX are shown in Fig. 6 and 7 respectively. The regression equation was found to be Y=43.07 x with correlation coefficient is r^2=0.999 which indicates this method has good linearity. The representative chromatograms indicating the SPFX are shown in Fig. 8 to 12. The linearity of the graph is shown in (Fig. 13).

The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo with sample peak. They do not disturb the elution or quantification of SPFX, furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 4. Precision was studied to find out intra and inter day variations in the test methods of SPFX for the three times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2) indicates that the proposed method is quite precise and reproducible; the results were shown in Table 5. Recovery studies of the drug were carried out for the accuracy parameter at three different concentrations levels i.e. multiple level recovery studies. A known amount of SPFX standard was added into pre-analyzed sample and subjected them to the proposed HPLC method. The percentage recovery was found to be within the limits as listed in Table 6. Generally the mean percentage recovery of SPFX at each level was not less than 99% and not more than 101%. In this case percentage recovery of SPFX was found to be in the range of 99.20 to 99.63%. The method precision was done and the low %RSD (0.116) values indicates that the proposed method which was in good agreement with precision. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, λ max, mobile phase composition etc., it was observed that there were no marked changes in the chromatograms. Infact the parameters are within the limit which indicates that the method has robustness and suitable for routine use. The Robustness results are presented in Table 7.The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) was 0.132 µg/ml and the limit of quantitation (LOQ) was 0.401 µg/ml which shows that this method is very sensitive. The results are presented in Table 8.

Table 1: optimized chromatographic conditions and system suitability parameters for proposed method.

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 X 250mm, 5µm)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Buffer: Acetonitrile (70:30 v/v)
Mobile phase	Buffer: ACN (70 : 30 v/v)
Flow rate	1ml/min.
Detection wave length	By UV at 291nm.
Run time	10 minutes
Column back pressure	118-119(kg/cm^2)
Temperature	Ambient temperature(25^oC)
Volume of injection loop	20(µl)
Retention time	5.497 min
Theoretical plates[th.pl] (Efficiency)	14711
Theoretical plates per meter[t.p/m]	294226
Peak asymmetry	1.143

Table 2: Linear regression data, precision of the proposed method of sparfloxacin:

Parameter	Method
Detection wavelength(λ max)	By UV at 293nm
Linearity range (µg/ml)	1-10µg/ml
Regression equation (Y=a+bc)	Y=43.07X
Slope(b)	43.07
Intercept(a)	0
Standard deviation of slope (S_b)	0.729008
Standard deviation of intercept (S_a)	2.427781
Standard error of estimation (Se)	1.50016085
Correlation coefficient (r)	0.999
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.116543
Limit of detection (LOD)	0.132 µg/ml
Limit of quantitation (LOQ)	0.401 µg/ml
Percentage range of errors* (Confidence limits) 0.005significance level 0.001 significance level	 0.12232 0.191838

***** Average of 6 determinations.

Table 3: Assay results of sparfloxacin formulations

Formulations	Labeled amount(mg)	Amount found(mg)	% Assay ±RSD*
Sparcip	100	99.43	99.43±0.11
Spardac	200	199.24	99.62±0.09

***** Average of 6 determinations.

Table 4: Specificity study

Name of the solution	Retention time in min.
Blank	No peaks
Sparfloxacin	5.497min.

Table 5: Results of Intraday and Interday precision study:

Sample	Injection number	Intraday precision	Interday precision
		Peak area	Peak area
Sparfloxacin	1	430.45	429.64
	2	429.05	430.33
	3	430.11	430.19
	4	430.27	429.27
	5	429.69	428.45
	6	430	430.39
	Mean	429.928	429.7117
	Standard deviation	0.501055	0.756747
	% RSD acceptance criteria 2.0)	0.116543	0.176104

Table 6: Recovery data of the proposed sparfloxacin RP-HPLC method.

Concentration level	Amount added (µg/ml)	Amount found (µg/ml)	Mean %Recovery ± SD*	%RSD #
	50	49.3		
50%	50	50	99.2±0.36	0.72
	50	49.5		
	100	99.95		
100%	100	98.89	99.61±0.62	0.62
	100	100		
	150	149.75		
150%	150	150	99.63±0.77	0.51
	150	148.55		

*SD is standard deviation; # %RSD is percentage of relative standard deviation.

Table 7: Robustness results of Sparfloxacin

S. No	Parameters	Optimized	Used	Retention time (t_R)	Peak asymmetry
1.	Flow rate (±0.2)	1 ml/min	0.8	5.535	1.155
			1	5.497	1.143
			1.2	5.421	1.147
2.	λ max (±5)	293nm	288	5.501	1.150
			293	5.497	1.143
			297	5.495	1.151
3.	Mobile phase composition (±5)	(Buffer: acetonitrile) 70:30	65:35	5.525	1.185
			70:30	5.497	1.143
			75:25	5.494	1.145

Table 8: Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Limit of Detection(LOD)	0.132µg/ml
Limit of Quantitation(LOQ)	0.401µg/ml

Table 9: Chromatogram results of proposed combination of fluoroquinolones

Name of the Compound	Retention time(t_R)	Assymmetry	Efficiency (theoretical plates)	Resolution
Levofloxacin	3.613	1.412	12306	—
Sparfloxacin	5.500	1.069	14729	12.143
Balofloxacin	6.257	1.063	15933	3.998

Table 10: Individual chromatogram results of three fluoroquinolone standards.

Name of the Compound	Retention time(t_R)	Assymmetry	Efficiency (theoretical plates)
Levofloxacin	3.607	1.333	12261
Sparfloxacin	5.497	1.143	14711
Balofloxacin	6.253	1.065	14112

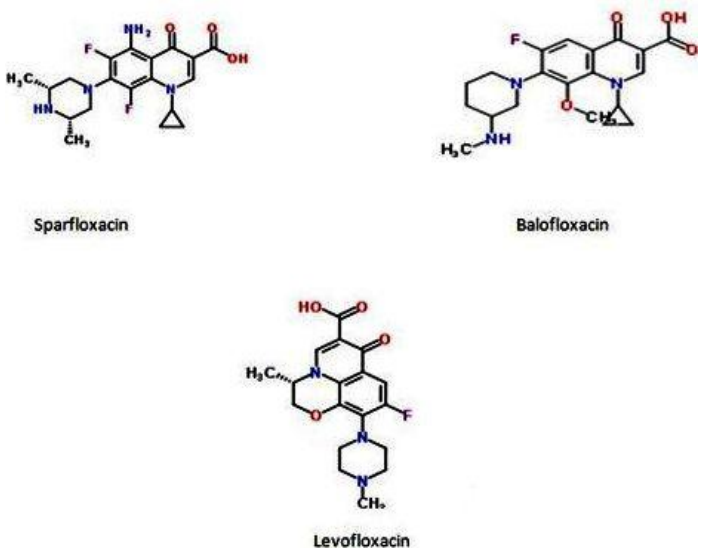

Fig. 1: Chemical structures of fluoroquinolones investigated in this study.

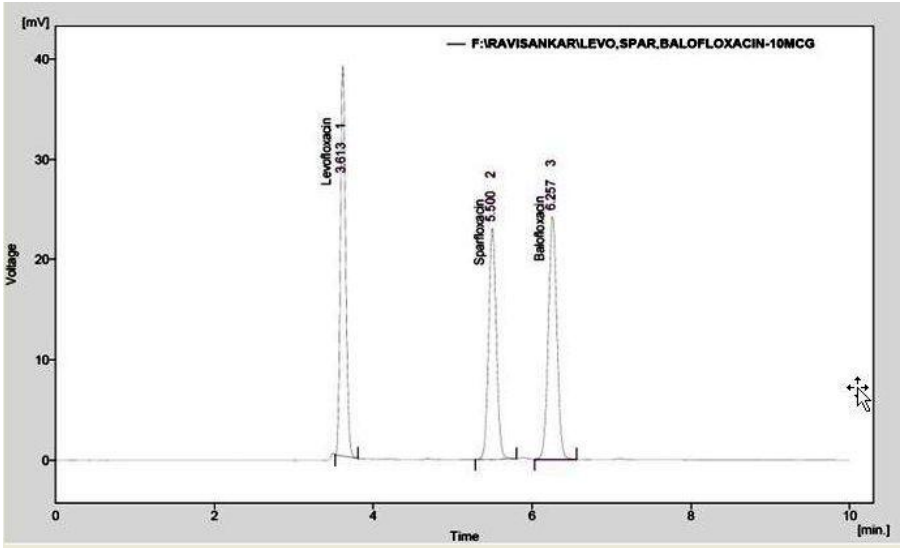

Fig. 2: standard chromatogram of mixture of Fluoroquinolone

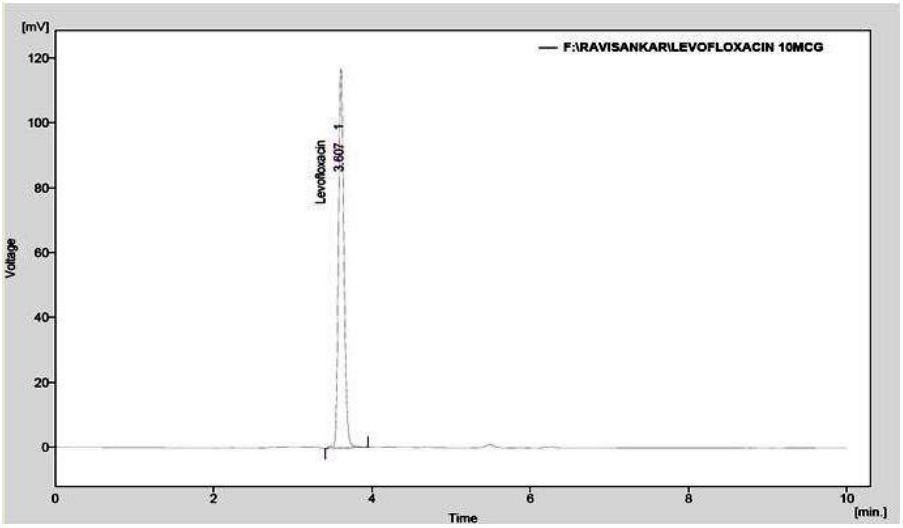

Fig.3: Standard chromatogram of Levofloxacin 10 µg/ml

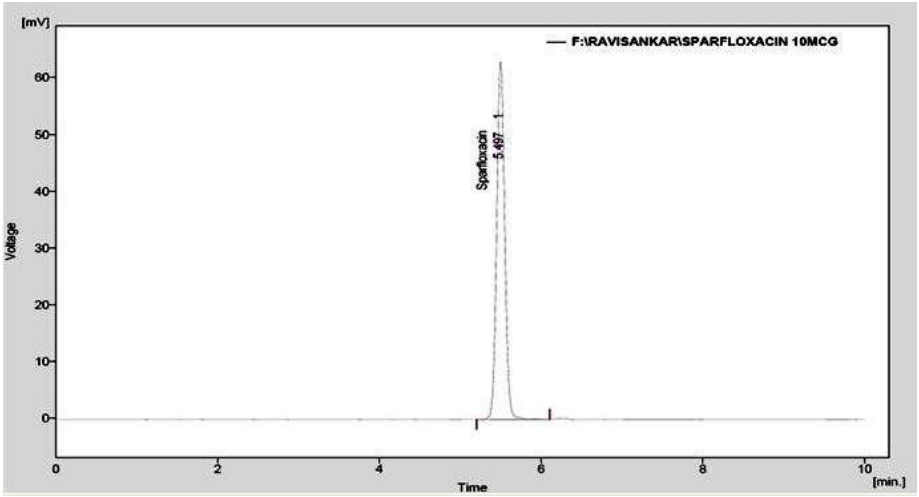

Fig. 4: Standard chromatogram of Sparfloxacin 10 µg/ml

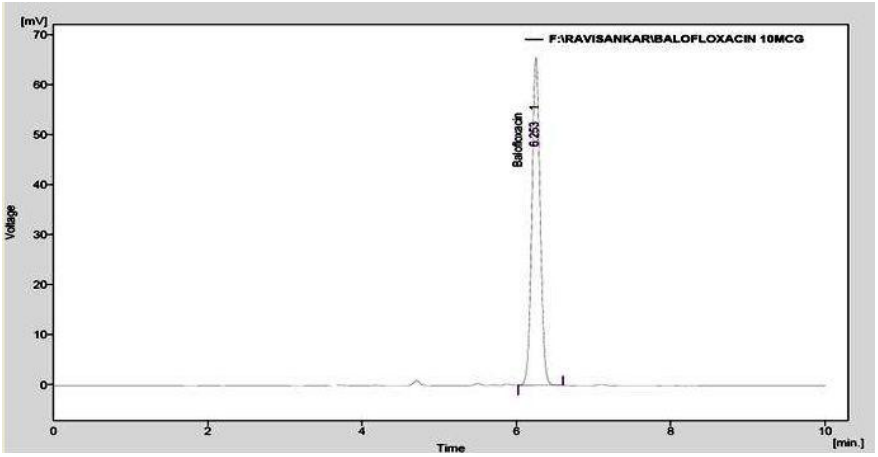

Fig. 5: Standard chromatogram of balofloxacin 10 µg/ml

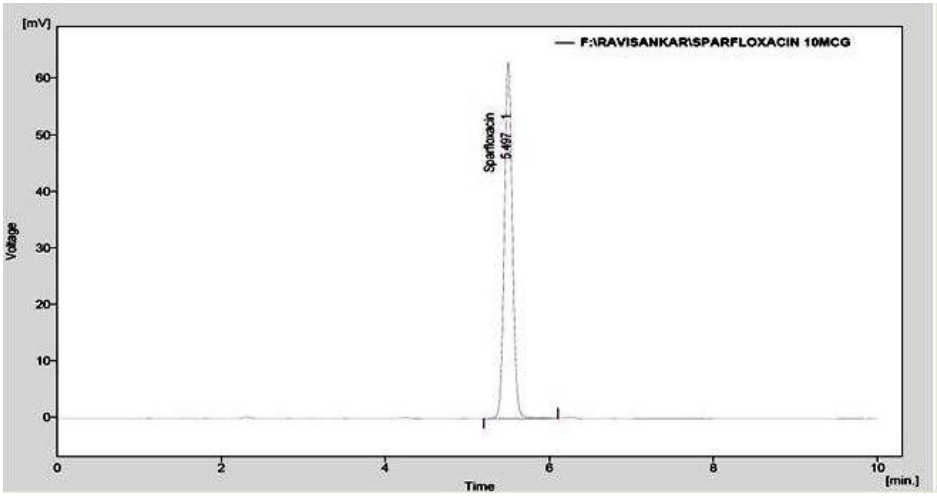

Fig.6 : A typical chromatogram of sparfloxacin standard

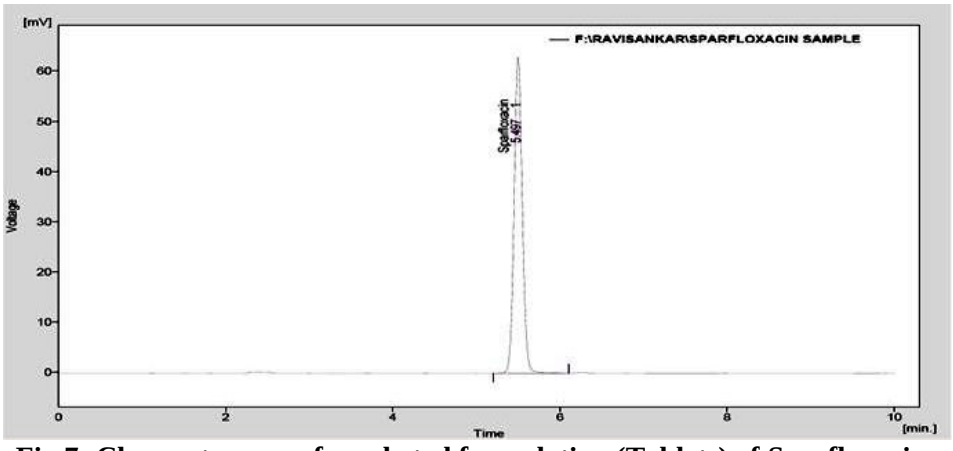

Fig.7: Chromatogram of marketed formulation (Tablets) of Sparfloxacin.

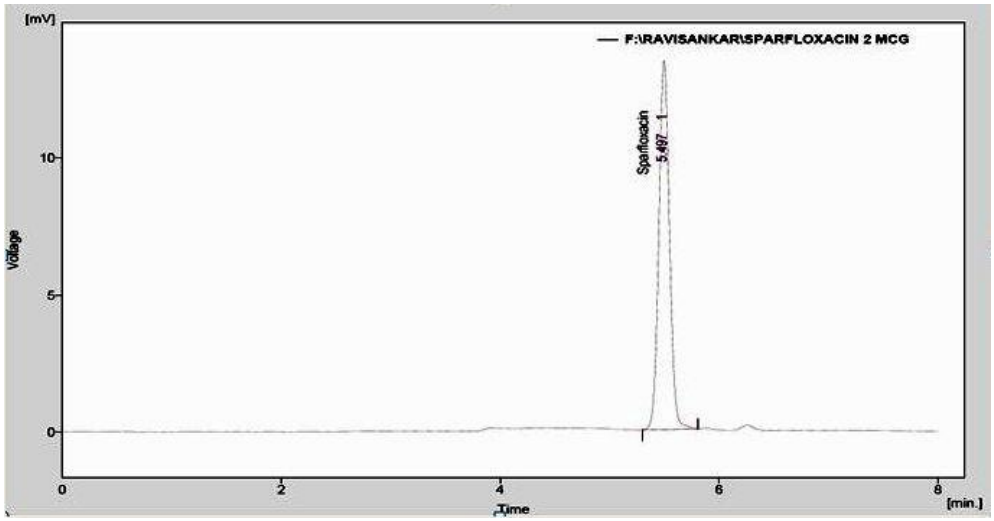

Fig. 8: Standard chromatogram of Sparfloxacin (2 µg/ml)

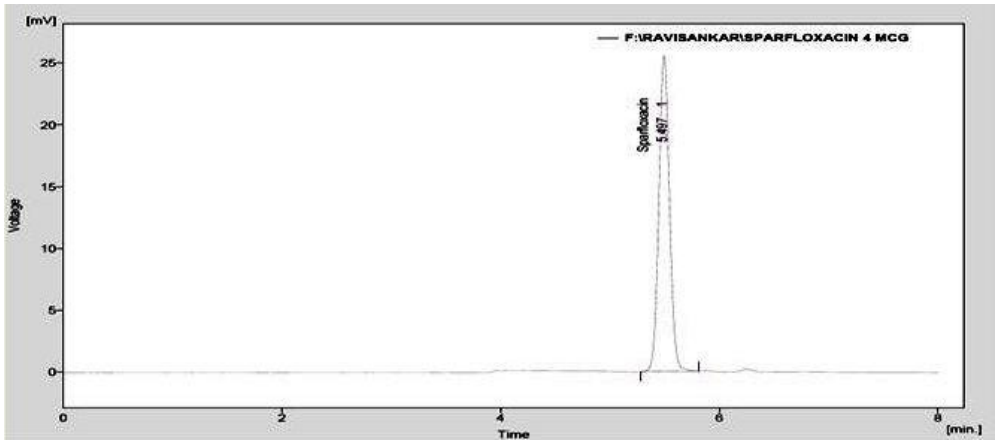

Fig. 9: Standard chromatogram of sparfloxacin (4µg/ml)

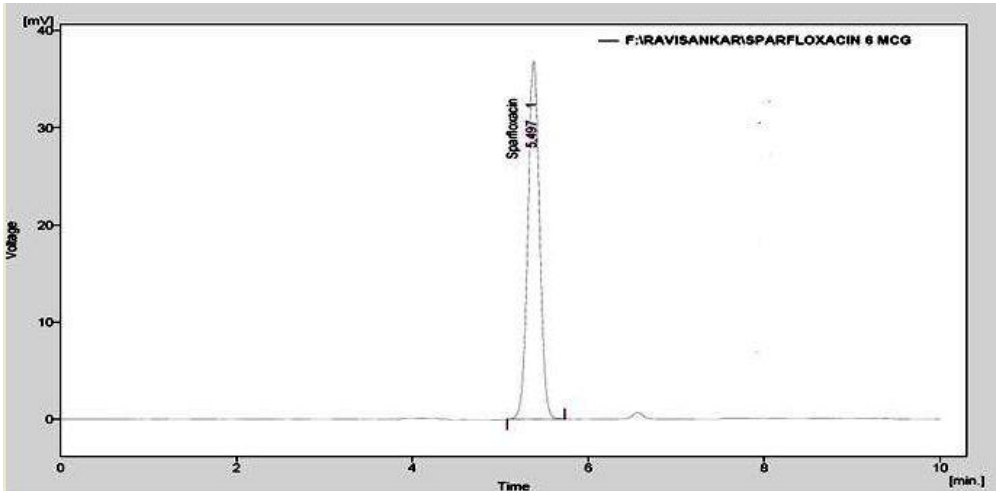

Fig. 10: Standard chromatogram of sparfloxacin(6 µg/ml)

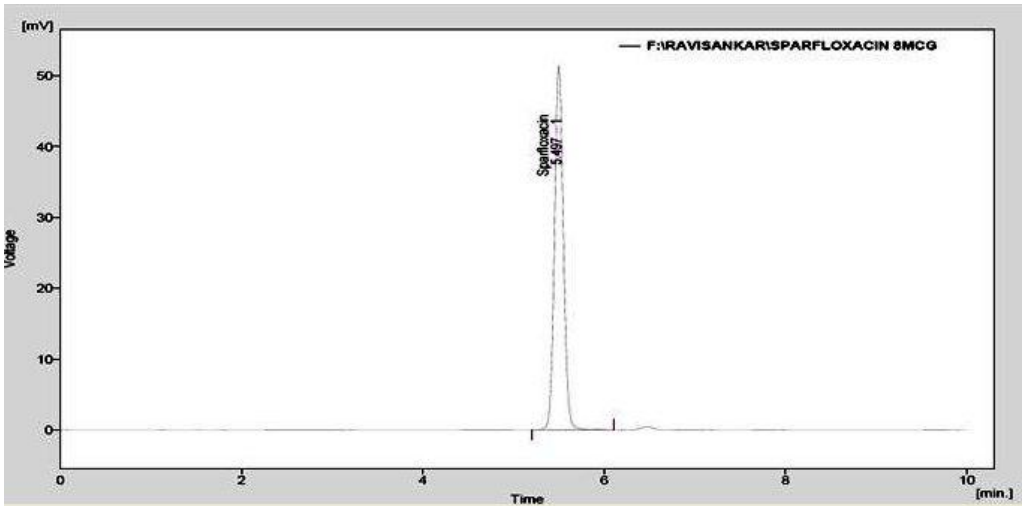

Fig.11: Standard chromatogram of sparfloxacin (8 µg/ml)

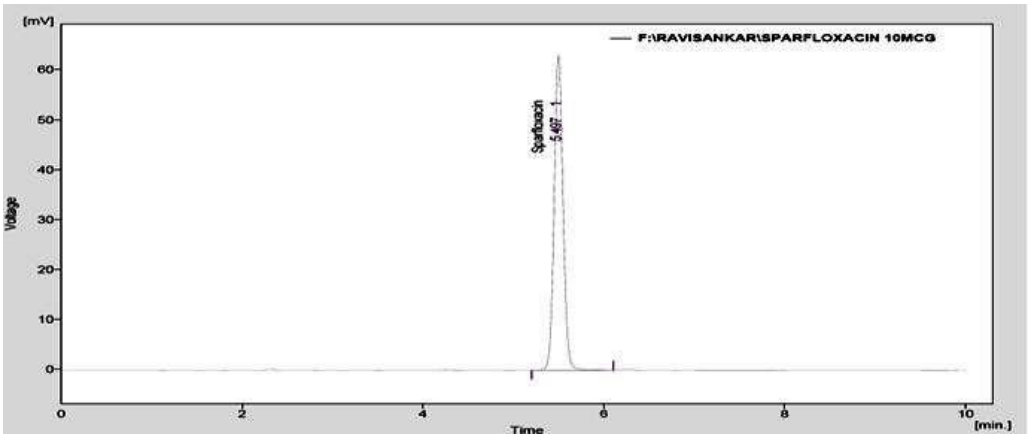

Fig. 12: Standard chromatogram of sparfloxacin (10µg/ml)

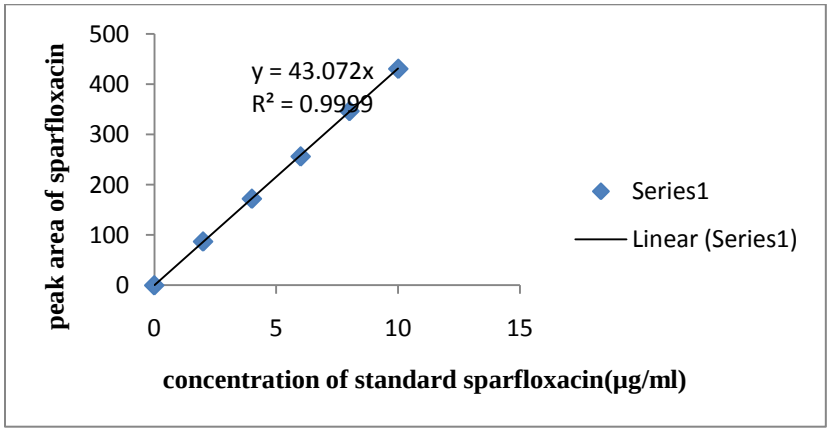

Fig. 13: Calibration plot of sparfloxacin:

4.CONCLUSION

A New validated RP-HPLC method has been developed for the quantitative determination of SPFX in tablet dosage forms in bulk and pharmaceutical dosage forms was established. The method was completely validated shows satisfactory results for all the method validation parameters tested and method was free from interference of the other active ingredients and additives used in the formulation. Infact results of the study indicate

that the developed method was found to be simple, reliable, accurate, linear, sensitive, economical, and reproducible and have short run time which makes the method rapid. Hence it can be concluded that this method may be employed for the routine quality control analysis of SPFX in active pharmaceutical ingredient (API) and pharmaceutical preparations. The method was successfully applied to sparfloxacin pharmaceutical dosage forms. It can also be applied for routine analysis of either one or of any combinations of these drugs in dosage forms.

5. ACKNWOLEDGEMENT

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