Original Article

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Intended high-performance liquid chromatography procedure for the quantification of norfloxacin and its potential impurities in active pharmaceutical ingredient and tablet dosage forms

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ABSTRACT

Objectives: Norfloxacin (NFXC) is an active pharmaceutical ingredient belongs to 4-quinolone antibacterial agent used for the prophylaxis of Gram-positive and Gram-negative bacterial infections. The present work aimed to develop a reliable stability-indicating reversed-phase high-performance liquid chromatography method for the separation and quantification of NFXC along with its synthetic impurities A and B. Results: High resolution and precise results were achieved on waters C18 Column (250 mm × 4.6 mm; 3.5 μm particle size) with a mobile phase of methanol, 0.01M sodium perchlorate (pH 3.1) in the ratio of 15:85 (v/v) at a flow rate of 0.8 ml/min in isocratic mode. Eluents were detected using ultraviolet detector at 220 nm. All the validation parameters are under the acceptance limit. Forced degradation studies were carried under acidic, basic, oxidative, photolytic, and thermal conditions. NFXC was found to be stable in all the stress conditions and proposed method can separate the known and unknown impurities. **Conclusion:** The developed method was found to be simple, precise, accurate, stable and was found to be suitable for the routine analysis of NFXC and its impurities A and B in bulk and pharmaceutical formulations.

INTRODUCTION

Norfloxacin (NFXC) (1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid) is a broad-spectrum antibiotic drug belongs to a group of medicines called quinolone antibiotics, especially 4-quinolone antibacterial agents.[1] NFXC used in the prophylaxis of bacterial infections in cirrhotic patients with gastrointestinal hemorrhage.[2] NFXC is a fluoroquinolone antimicrobial agent used for the treatment of uncomplicated and complicated urinary tract infection.[3] NFXC is a derivative of nalidixic acid, but it possesses greater antibacterial activity against Gram-positive and Gram-negative bacteria than nalidixic acid.[4]

DNA gyrase is the essential enzyme for DNA replication. NFXC works by inhibiting the subunit of DNA gyrase.[5] No abnormal side effects were observed for NFXC. The side effects occur with the use of NFXC was common such as nausea, diarrhea, heartburn, dizziness, stomach cramps, headache, and weakness.[6]

Literature survey reveals that few methods were high-performance liquid chromatography (HPLC) analytical methods were available for the analysis of NFXC in pharmaceutical formulations[7-9] and biological samples.[10] Rao and Nagaraju[11] developed a HPLC method for the determination of NFXC along with synthetic impurities, impurity A, 7-chloro-6-fluoro-1-methyl-4-oxo-1,4-dihydro-3-qui-nolinecarboxylic acid and ethyl-7-chloro-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylate (CAT). CAT and EAT are constitutional isomers. No methods were detected so far for the analysis of NFXC along with its synthetic impurities, impurity A [7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] and

impurity B [7-[(2-aminoethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid]. Hence, we attempted to develop a reversed-phase-HPLC method for the separation and analysis of NFXC and its impurities A and B. The molecular structure of NFXC, impurities A and B were given in Figure 1.

MATERIALS AND METHODS

Instrumentation

The separation and estimation of NFXC with impurities A and B was done on PEAK HPLC (India) system. Mobile phase was pumped into column using LC-P7000 isocratic pump. 20 μl fixed volume sample was injected for the analysis using rheodyne injector (model 7725) with fixed 20 μl loop. Variable wavelength programmable ultraviolet (UV)/visible detector was used for detecting the compounds. The detector response signals were monitored and integrated using WS-100 Workstation software (Cyberlab, USA). Samples were injected using Hamilton (USA) manual HPLC syringe. Double beam UV-visible spectrophotometer (Teccomp UV-2301 - India) was used for spectral analysis. Denver electronic analytical balance (SI-234) was used for weighing the standards and samples. pH of the mobile phase was adjusted using Systronics (India) digital pH meter (Sr No S 1326).

Chemicals and Reagents

NFXC active pharmaceutical ingredient (API) and its two impurities A and B were obtained from Dr. Reddy's Laboratories Private Limited, India. The marketed formulation of NFXC (Gramoneg© - 500 mg) was purchased in local pharmacy. Laboratory reagent grade sodium perchlorate and perchloric acid were purchased from SD Fine-Chem Limited, Mumbai. HPLC grade methanol, acetonitrile, and water were purchased from Merck chemicals, Mumbai. 0.2 μ nylon membrane filter papers were used for filtration of samples and mobile phase and were purchased from millipore (India).

Preparation of NFXC, Impurities A and B Solutions

About 100 mg of standard drug NFXC was weighed accurately and was dissolved in 50 ml methanol. Mixed the solution till the drug dissolved completely in methanol. Then, the final volume was made up to 100 ml with methanol. Filter the final solution using 0.2 μ nylon membrane filter paper. A standard stock solution concentration of 1000 μg/ml was obtained. Necessary dilutions required for the analysis were prepared from this stock solution.

Preparation of Formulation Solution

The marketed formulation tablets of NFXC (Gramoneg©- 500) were finely powdered using clean, dry mortar, and pestle. An amount of the uniform fine powder equivalent to 100 mg of NFXC standard was weighed accurately and was dissolved in 75 ml methanol. Keep the solution in an airtight 100 ml volumetric flask and was kept in a rotatory orbital shaker to 24 h. Then, it was filtered and make up to the mark with methanol. Sample solution concentration of 1000 μg/ml was obtained. Then, the solution was diluted properly to get a final sample concentration of 300 μg/ml.

METHOD DEVELOPMENT

Method development was instantiated by checking the solubility of standard drug and both impurities. NFXC, impurities A and B solutions were soluble in methanol. Hence, methanol was used as diluents for the analysis. Spectrophotometer analysis was carried for the determination of suitable common wavelength for NFXC, impurities A and B.

A concentration of 10 μg/ml of NFXC, impurity A and impurity B were scanned in the region of 800 nm–400 nm using UV/visible spectrophotometer. The wavelength maxima were found to be 261 nm for NFXC [Figure 2], 276 nm for impurity A [Figure 3], and 220 nm for impurity B [Figure 4]. The isoabsorption wavelength of NFXC, impurities A and B were found to be 220 nm. Hence, 220 nm was found to be suitable for the analysis of NFXC and its impurities A and B simultaneously by HPLC-UV detection method.

The optimization of suitable mobile phase was carried based on the chemical properties of the three molecules in the study. Based on the properties and available literature, method has development have been initiated with ionic buffer, i.e., sodium perchlorate salt as aqueous solvent and methanol as organic modifier.

Trial and error method was followed for the optimization of chromatographic conditions. Method development was initiated with low volume of 0.01 M sodium perchlorate and higher volume of organic modifier methanol in the ratio of 25:75 (v/v) on C18 column (100 mm × 4.6 mm × 3.5 μ). In this condition, no clear baseline was observed. Further, the pH of the mobile phase was adjusted to pH 3.1 gives a clear baseline, but the resolution was found to be very poor and similar retention times were observed for NFXC, impurities A and B. Short length column 100 mm influencing the interaction time of molecules with stationary phase.

Method optimization was obtained in between the three compounds NFXC, impurity A, and impurity B [Figure 5]

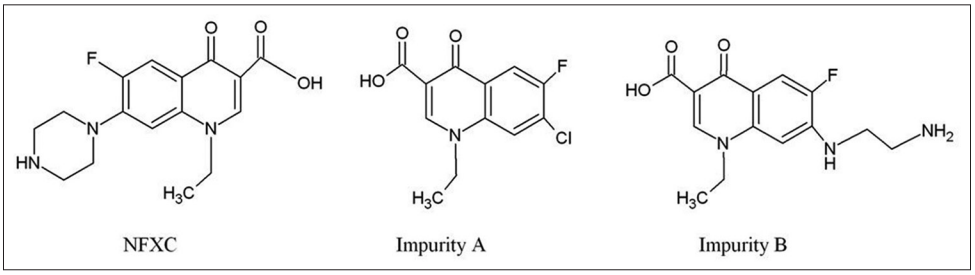


Figure 1: Molecular structures of norfloxacin and impurities in the study

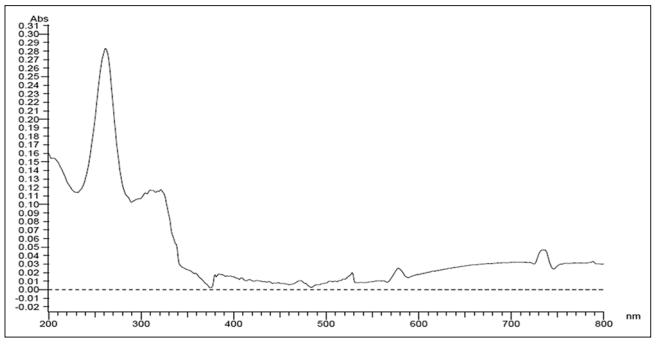


Figure 2: Ultraviolet absorption spectrum of norfloxacin

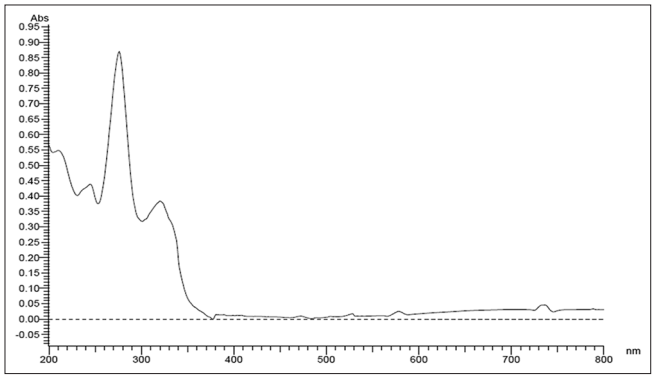


Figure 3: Ultraviolet absorption spectrum of impurity A

with mobile phase composition of methanol, 0.01 M sodium perchlorate (pH 3.1) in the ratio of 15:85 (v/v) at a flow rate of 0.8 ml/min. Waters C18 column (250 mm × 4.6 mm; 3.5 μm) and 220 nm, 20 μl injection volume was selected for the analysis.

In the optimized condition, peak purity of NFXC was studied separately using PDA detector. The purity angle of NFXC was found to be less than purity threshold confirms that the peak purity for NFXC peak was within acceptance criteria. The peak purity was found to be 99.3% and the peak purity index was found 0.993 which was within the acceptance limit of ≥0.990.

METHOD VALIDATION

Method validation was performed as per ICH guidelines for NFXC and synthetic impurities impurity A and impurity B.[12]

Specificity and System Suitability

The chromatogram of standard and placebo solution obtained in the optimized method was confirm the specificity. It was observed that no chromatographic detection was observed in the retention times of NFXC and both related substances for placebo analysis confirm that the method developed was found to be suitable for the separation and analysis of NFXC and its related impurities A and B.

The standard solution of NFXC and impurities at a concentration of 10, 20, and 30 μg/ml was prepared separately and was mixed in equal proportions separately. These solutions were analyzed triplicates in the developed method. The number of theoretical plates, tailing factor, and resolution factor was used for the determination of system suitability. The

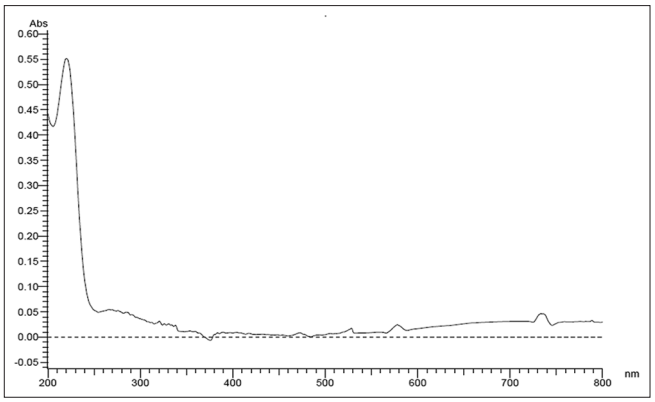


Figure 4: Ultraviolet absorption spectrum of impurity B

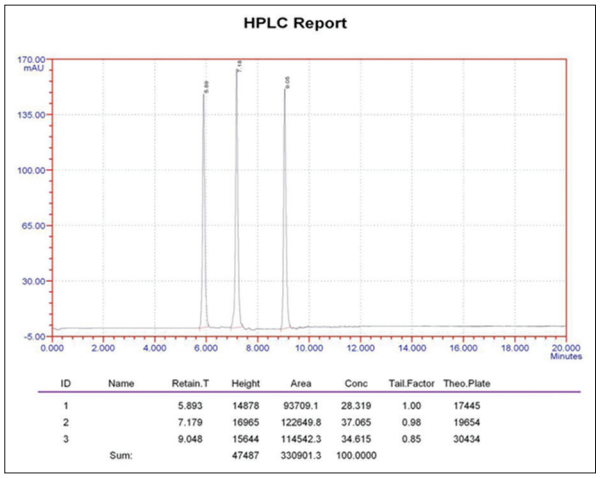


Figure 5: Standard chromatogram for norfloxacin (4 μg/ml)

results of system suitability [Table 1] confirm that the number of theoretical plates was found to be more than 2500, <2 tailing factor, and >2 resolution factor was observed for NFXC and impurities A and B. Hence, the method developed was found to be suitable and specific.

Relative Retention Time (RRT) and Relative Response Factor (RRF)

RRT determines the stability of the elution time of the impurities of NFXC in the developed method. RRF was used to determine the reproducible response of the pharmaceutical impurities in the developed method. RRF also helpful for the determination of actual amount of impurities present in pharmaceuticals. The ratio between the signals obtained for impurity to the signal produced by the NFXC API in the same experimental condition was considered as RRF. Standard NFXC and impurities at a concentration of 10 μg/ml, 20 μg/ml, and 30 μg/ml were prepared and were mixed in equal volume. The solutions were analyzed in triplicates in the developed method and RRT and RRF were calculated. The RRT and RRF were found to be within the acceptance limit of <2 [Table 2] for both impurities A and B in all three concentration levels. Hence, reproducible retention time and response were observed in the developed method for the analysis of NFXC and impurities A and B.

Linearity

A 6 points calibration curve was constructed for NFXC and both impurities A and B. A combined solutions containing equal concentration of NFXC and impurities were prepared in different concentration range and the solutions were analyzed in triplicates in the optimized method. The response observed at the retention time (Tr) of compound was used for constructing the calibration. Calibration curve was constructed by taking response at Tr of compound on x-axis and concentration prepared on y-axis. Accurately correlated calibration curve was observed in the concentration range of 1–6 μg/ml for NFXC, impurities A and B. Correlation and regression results were given in Table 3 and calibration curve results were given in Table 4. Calibration curve was given in Figure 6 for NFXC and impurities A and B.

Recovery

The accuracy and recovery of the method developed for the analysis of NFXC was determined by spiked recovery method. 50%, 100%, and 150% concentrations were spiked for a fixed concentration of 2 μg/ml and the solution was analyzed in triplicates. The result obtained in spiking experiment was compared with the standard calibration curve values and % recovery and % relative standard deviation (RSD) of recovery was calculated. The % recovery was found to be in the range of 98–100. The % RSD was found to be 0.122, 0.305, and 0.147 in 50% spiked level, 0.106, 0.383, and 0.541 in 100%, and 0.170, 0.212, and 0.153 in 150% spiked level for impurity A, standard and impurity B, respectively. % RSD was found to be very less confirmed that the method developed for NFXC was very accurate. Table 5 summarizes the recovery results for NFXC.

Table 1: System suitability results for NFXC

Concentration (μg/mL)	Compound	RT (min)	Theoretical plates	Tailing factor	Resolution
10	Standard	6.90	81002	1.38	13.88
	Impurity A	5.61	67996	1.37	…
	Impurity B	8.82	154807	1.35	21.34
20	Standard	6.93	91722	1.42	14.03
	Impurity A	5.61	53276	1.36	…
	Impurity B	8.87	159953	1.48	21.49
30	Standard	6.87	91439	1.31	14.74
	Impurity A	5.49	47173	1.36	…
	Impurity B	8.85	145733	1.54	21.32

Three replicate measurements average values given in the table. NFXC: Norfloxacin

Table 2: RRT and RRF results

Concentration (μg/mL)	Compound	RT (min)	RRT	RF	RRF
10	Standard	6.90	…	10676	…
	Impurity A	5.61	0.81	11764	1.10
	Impurity B	8.82	1.28	8629	0.81
20	Standard	6.93	...	6288	…
	Impurity A	5.61	0.81	6644	1.06
	Impurity B	8.87	1.28	4616	0.73
30	Standard	6.87	…	4592	…
	Impurity A	5.49	0.80	4965	1.08
	Impurity B	8.85	1.29	3737	0.81

Three replicate measurements average values given in the table; RRT: Relative retention time, RRF: Relative response and keep

Table 3: Correlation and regression results for NFXC

Compound	Slope	Intercept	r^2
Standard	27234.67±222.28	45697.67±62.93	0.998
Impurity A	18458.00±263.50	37557.33±485.97	0.999
Impurity B	22858.67±76.27	44389.00±622.34	0.998

#Values given in table is the average±standard deviation of peak response in three replicate experiments. NFXC: Norfloxacin

Force degradation

Force degradation studies were used to identify and quantify known and unknown impurities in NFXC. Force degradation studies were performed and procedure was summarized in Table 6.

Results confirmed that stress condition does not influence the elution of standard drug NFXC. Both impurities A and B were observed in all the stress studies. In addition to these known impurities, two unknown impurities were detected. NFXC was very sensitive to basic condition, 7.187% degradation was observed in 5 h [Figure 7]. High % degradation about 11% was observed in photolytic degradation in 24 h [Figure 8]. The effect of other stress conditions was found to be nominal [Figures 9 and 10]. All force degradation studies results were tablets in Table 7.

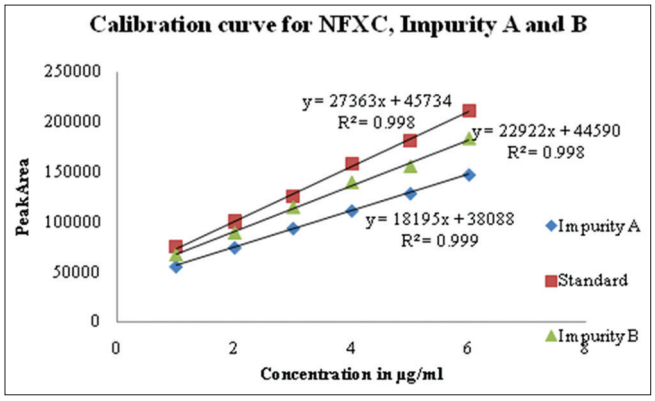


Figure 6: Calibration curve

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ are calculated theoretically based on the response factor of the NFXC, impurities A and B. S/N ratio approach was used for LOD and LOQ concentrations determination. LOD was established with 0.03 μg/ml, 0.01 μg/ml, and 0.01 μg/ml for NFXC, impurities A and B, respectively [Figure 11]. The LOD and LOQ results [Table 8] were confirmed the developed method has high sensitivity.

NFXC Drug Substance Analysis

NFXC drug substance was used to prepare 300 μg/ml solution and analyzed in the finalized procedure. Results were satisfactory. Chromatogram was represented in Figure 12. Method confirming that finalized method has high detectability. Hence, method can be applied for the identification and estimation of synthetic impurities in NFXC drug synthesis.

Formulation Analysis

Gramoneg© - 500 tablets, market sample was purchased for analysis. 300 μg/ml concentration sample was analyzed and spiked sample was analyzed with two impurities A and B spiking. No other additional impurities were detected in the market sample. Spiked sample was represented in Figure 13.

Table 4: Calibration curve results

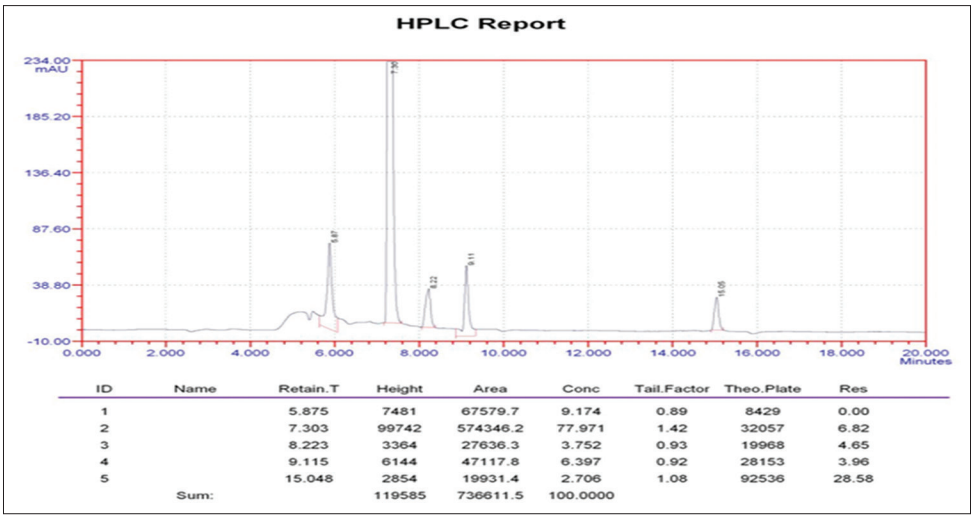
Concentration in µg/ml	Peak area obtained[#]		
	Standard	Impurity A	Impurity B
1	75008.00±168.88	56148.00±549.80	67169.33±723.08
2	99980.33±545.88	74573.00±142.17	89447.67±570.00
3	123727.70±1006.24	92111.00±1500.53	113535.30±1260.81
4	154502.70±2864.39	112166.30±920.52	137669.00±1561.52
5	180580±888.64	1296970±1208.98	156299.30±875.24
6	209883.30±1131.39	148269.70±1087.35	182241.00±1264.01

[#]Values given in table is the average±standard deviation of peak response in three replicate experiments

Table 5: Recovery results for NFXC in the developed method

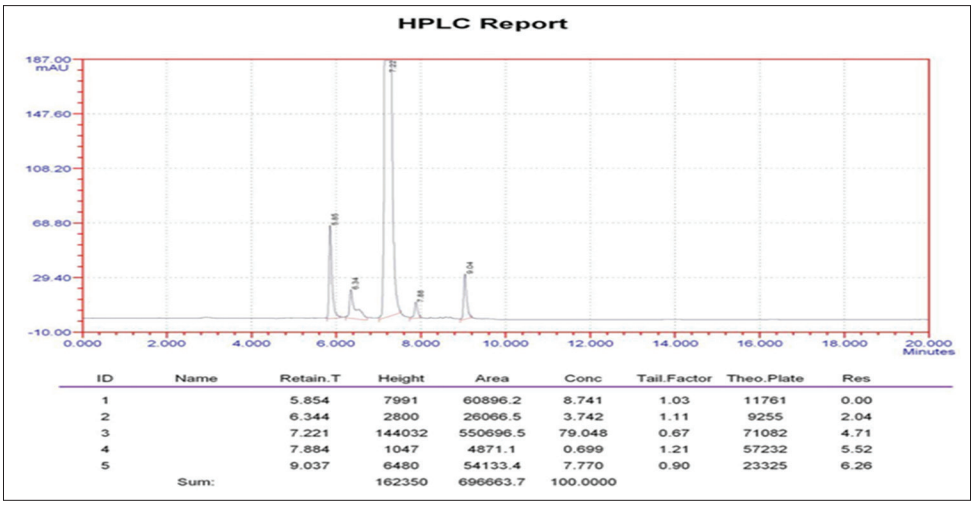
Recovery level	Compound	Concentration in µg/ml			Amount found	% recovered	% RSD of Recovery
		Target	Spiked	Final	Mean±SD	Mean±SD	
50%	Standard	2	1	3	2.956±0.009	98.522±0.301	0.305
	Impurity A	2	1	3	2.948±0.004	98.267±0.120	0.122
	Impurity B	2	1	3	2.962±0.004	98.733±0.145	0.147
100%	Standard	2	2	4	3.957±0.015	98.917±0.378	0.383
	Impurity A	2	2	4	3.927±0.004	98.183±0.104	0.106
	Impurity B	2	2	4	3.951±0.021	98.767±0.535	0.541
150%	Standard	2	3	5	4.914±0.0104	98.287±0.208	0.212
	Impurity A	2	3	5	4.926±0.008	98.527±0.168	0.170
	Impurity B	2	3	5	4.908±0.008	98.167±0.150	0.153

NFXC: Norfloxacin, RSD: Relative standard deviation, SD: Standard deviation



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		5.875	7481	67579.7	9.174	0.89	8429	0.00
2		7.303	99742	574346.2	77.971	1.42	32057	6.82
3		8.223	3364	27636.3	3.752	0.93	19968	4.65
4		9.115	6144	47117.8	6.397	0.92	28153	3.96
5		15.048	2854	19931.4	2.706	1.08	92536	28.58
	Sum:		119585	736611.5	100.0000			

Figure 7: Base degradation chromatogram (BH)



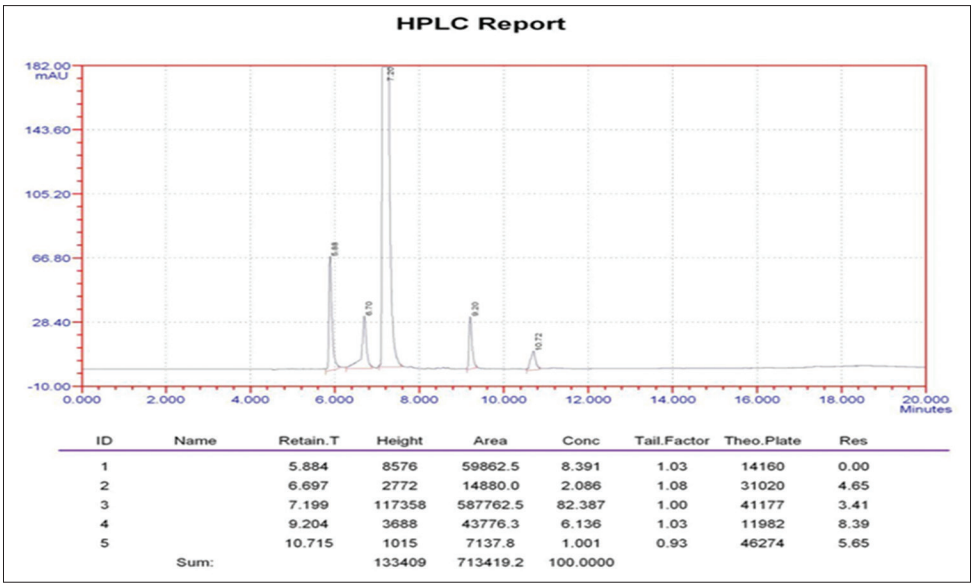
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		5.854	7991	60896.2	8.741	1.03	11761	0.00
2		6.344	2800	26066.5	3.742	1.11	9255	2.04
3		7.221	144032	550696.5	79.048	0.67	71082	4.71
4		7.884	1047	4871.1	0.699	1.21	57232	5.52
5		9.037	6480	54133.4	7.770	0.90	23325	6.26
	Sum:		162350	696663.7	100.0000			

Figure 8: Photolytic degradation chromatogram (PD 2)

Table 6: Forced degradation study procedure for NFXC

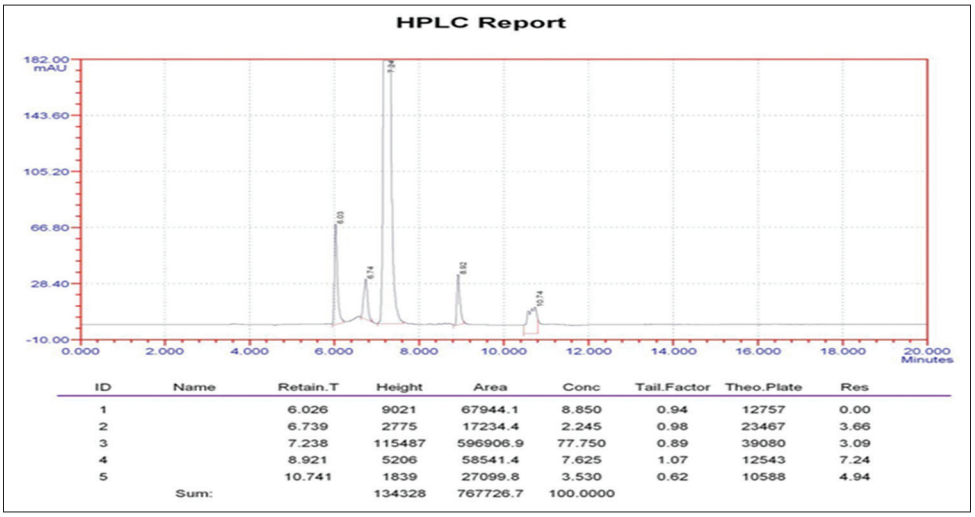
Degradation type	Experimental conditions	Sample code
Acid hydrolysis (AH)	50 mg of drug was mixed with 50 ml of 0.1N HCl solution. The solution was neutralized and diluted up to standard concentration and was analyzed in the developed method condition	5 h (AH 1) 24 h (AH 2)
Base hydrolysis (BH)	50 mg of drug was mixed with 50 ml of 0.1N NaOH solution. The solution was neutralized and diluted up to standard concentration and was analyzed in the developed method condition	5 h (BH)
Oxidative degradation (OD)	50 mg of drug was mixed with 50 ml of 3% peroxide solution. The solution was neutralized and diluted up to standard concentration and was analyzed in the developed method condition	5 h (OD 1) 24 h (OD 2)
Photolytic degradation (PD)	50 mg of drug sample was kept in UV light (254 nm). After the selected time of light expose, the drug solution was prepared and was analyzed	5 h (PD 1) 24 h (PD 2)
Thermal degradation (TD)	50 mg of drug sample was kept in oven at 60°C. After the selected time of light expose, the drug solution was prepared and was analyzed	5 h (TD 1) 24 h (TD 2)

NFXC: Norfloxacin



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		5.884	8576	59862.5	8.391	1.03	14160	0.00
2		6.697	2772	14880.0	2.086	1.08	31020	4.65
3		7.199	117358	587762.5	82.387	1.00	41177	3.41
4		9.204	3688	43776.3	6.136	1.03	11982	8.39
5		10.715	1015	7137.8	1.001	0.93	46274	5.65
	Sum:		133409	713419.2	100.0000			

Figure 9: Acid hydrolysis chromatogram (PD 2)



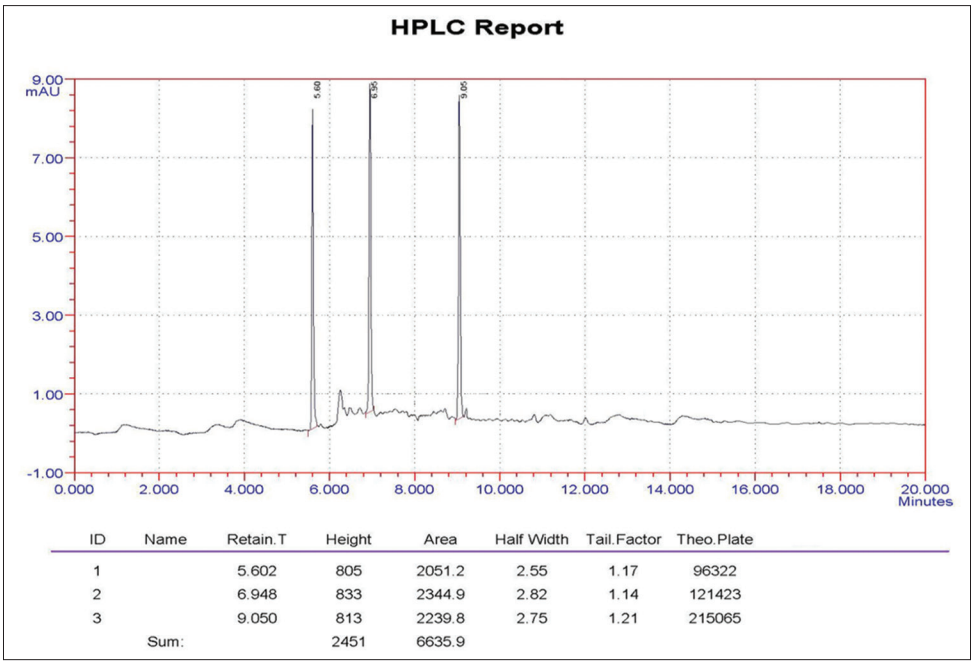
ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1		6.026	9021	67944.1	8.850	0.94	12757	0.00
2		6.739	2775	17234.4	2.245	0.98	23467	3.66
3		7.238	115487	596906.9	77.750	0.89	39080	3.09
4		8.921	5206	58541.4	7.625	1.07	12543	7.24
5		10.741	1839	27099.8	3.530	0.62	10588	4.94
	Sum:		134328	767726.7	100.0000			

Figure 10: Acid hydrolysis chromatogram (AH 2)

Table 7: Forced degradation study results for NFXC

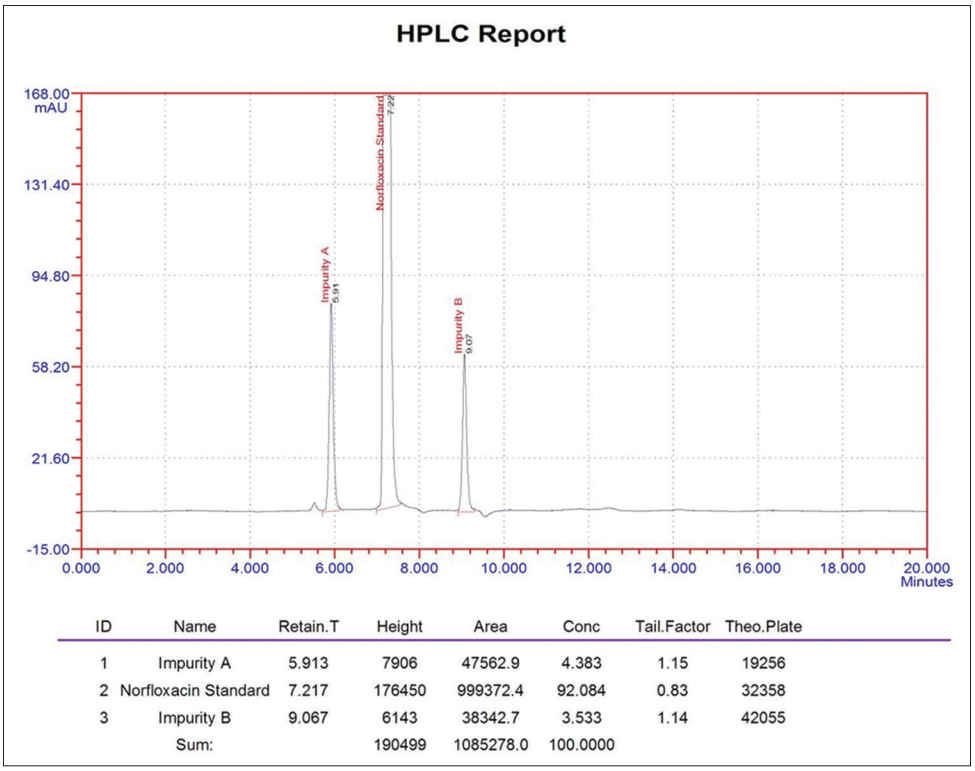
Condition	No of addition peaks observed	Area obtained	% Obtained	% degradation
Standard	….	618822	-	-
AH 1	0	600497	97.039	2.961
AH 2	2	587762	94.981	5.019
BH 1	2	574346	92.813	7.187
OD 1	0	608235	98.289	1.711
OD 2	1	581180	93.917	6.083
PD 1	1	575315	92.969	7.031
PD 2	2	550696	88.991	11.01
TD 1	0	607299	98.138	1.862
TD 2	2	596906	96.458	3.542

NFXC: Norfloxacin, AH: Acid hydrolysis, BH: Base hydrolysis, OD: Oxidative degradation, PD: Photolytic degradation, TD: Thermal degradation



ID	Name	Retain.T	Height	Area	Half Width	Tail.Factor	Theo.Plate
1		5.602	805	2051.2	2.55	1.17	96322
2		6.948	833	2344.9	2.82	1.14	121423
3		9.050	813	2239.8	2.75	1.21	215065
	Sum:		2451	6635.9			

Figure 11: Limit of detection chromatogram of norfloxacin



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate
1	Impurity A	5.913	7906	47562.9	4.383	1.15	19256
2	Norfloxacin Standard	7.217	176450	999372.4	92.084	0.83	32358
3	Impurity B	9.067	6143	38342.7	3.533	1.14	42055
	Sum:		190499	1085278.0	100.0000		

Figure 12: Norfloxacin drug substance + impurities spiked chromatogram

In literature, few methods were reported previously for the estimation of NFXC in pharmaceutical formulations. The method described by Sharma *et al*., 2014; Ashok *et al*., 2014, and Paulo *et al*., 2009, were found to be a routine assay methods and they did not study the stability of NFXC in stress conditions and the method cannot be applicable for the separation and estimation of impurities. Lucas *et al*., 2013, studied the stability of the drug in different stress degradation conditions. However, the method fails to analysis the impurities. The method developed by Mahmood *et al*., 2010, and Nageswara *et al*., 2004, were for the estimation of drug in plasma and synthetic impurities, respectively, and these methods were not in our area of study. Hence, the method developed here was found to be the simple and novel

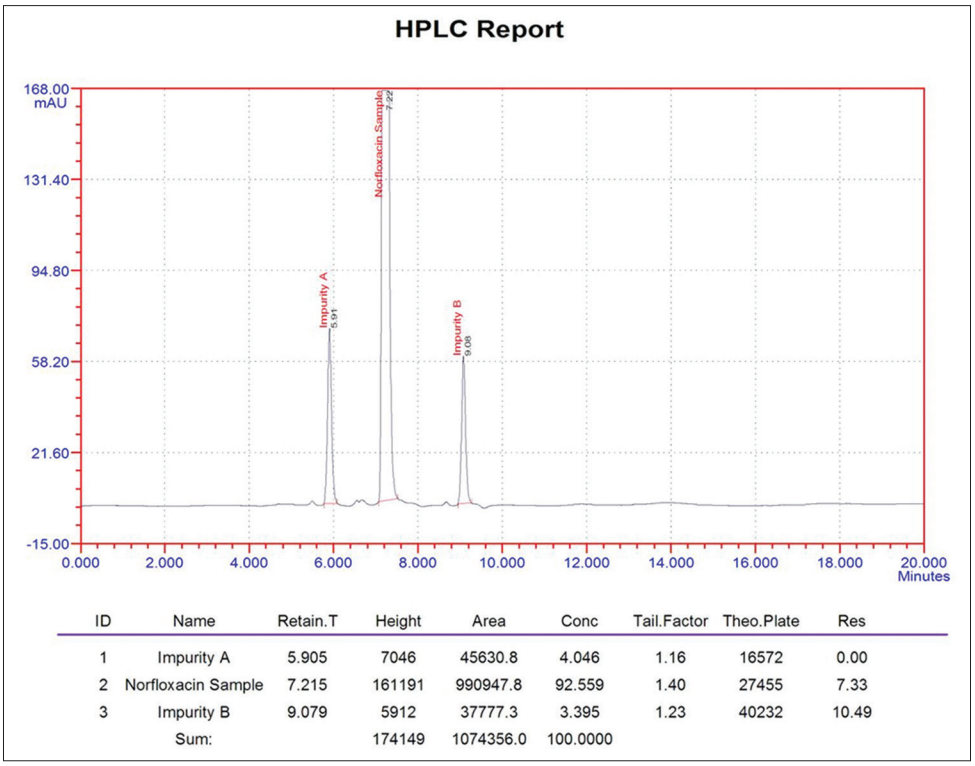


Figure 13: Gramoneg© - 500 tablets+ impurities spiked sample

Table 8: Sensitivity results

Compound	Theoretical value		Instrumental results	
	LOQ (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	LOD (µg/ml)
Standard	0.01	0.003	0.1	0.03
Impurity A	0.005	0.0015	0.04	0.01
Impurity B	0.02	0.004	0.04	0.01

LOD: Limit of detection, LOQ: Limit of quantification

method for the separation, qualitative determination and quantification of NFXC and its potential related compounds A and B.

CONCLUSION

Isocratic HPLC procedure was developed for NFXC and its impurities (A and B). Method was validated as per ICH Q2 guideline. All method validation parameter results found within the acceptance limit. Method is simple, accurate, and reliable and can be applied for drug substance synthesis and tablet dosage forms. Methods for the separation and estimation of NFXC and its impurities A and B were not reported previously, and hence, the method found to be novel and suitable for stability testing, impurity profile analysis of NFXC.

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