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Development and validation of RP-HPLC method for quantitative estimation of related substances in gatifloxacin drug substance

ABSTRACT

The analysis of improved RP-HPLC method for the separation and quantification of Gatifloxacin and its impurities are described. Samples are analysed by means of reverse phase (RP-HPLC) using an Zorbax Eclipse C18, 50 x 4.6 mm5µm and the mobile phase consists of two Channels A and B.Channel-A:0.1% Trifluoroacetic acid buffer and Channel-B:Acetonitrile. The flow rate is 1.0 ml/min. The column temperature was maintained at 35°C and sample temperature was maintained at 25°C, injection volume 10µLand wavelength fixed at 220nm UV-detection. The developed LC method was validated with respect to specificity, precision, linearity, ruggedness and robustness. Validation study compared as per ICH guideline.

Keywords: *Gatifloxacin, determination of related substances, liquid chromatography*

1. INTRODUCTION

Gatifloxacin, 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxoquinoline-3-carboxylic acid, is a fourth-generation antibiotic of the fluoroquinolone family which inhibits the bacterial enzymes DNA gyrase and topoisomerase IV [1]. The fluoroquinolones are quinolones with fluorine at position 6 of the naphthyridine ring [2].Published structure–activity data show that the fluorine atom helps broadens their range of activity against both

Gram-negative and Gram-positive pathogens [3,4]. A simple reversed-phase high-performance liquid chromatographic(LC) method for assessment ofthe stability of Gatifloxacin in human plasma has been developed and validated [5]. A europium-sensitized fluorescence spectrophotometric method using an anionic surfactant, sodium dodecyl benzene sulphonate (SDBS), has also been developed for analysis of Gatifloxacin [6]. Papers have been published describing methods for analysis of Gatifloxacin in combination with ornidazole in tablet dosage forms [7].

Most of the methods reported involve troublesome mobile phases (buffers) and difficult detection methods (fluorescence). There are no published reports of methods foranalysis of Gatifloxacin in bulk samples inthe presence of degradation products and process-related impurities.

The objectives of the research work reported in this paper were to develop a suitable stability-indicating LC method for analysis of Gatifloxacin and to validate the method for specificity, LOD,LOQ, linearity, precision, accuracy, and robustness to show the stability-indicating power of the method and to ensure compliance with ICH Guidelines [8-12].The molecule structure is shown in Figure: 1

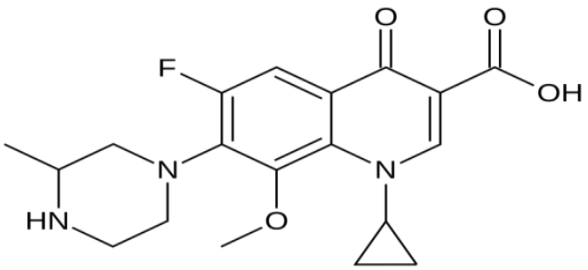

Figure: 1 Chemical structure of Gatifloxacin

Slika 1. Hemijska struktura Gatifloxacin-a

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and

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formulations is one of the most challenging tasks. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products. For these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Trifluoroacetic acid and Acetonitrile was procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received.

Instrumentation

Chromatographic separation was achieved by using an Waters e2695, Empower^3 software using, Zorbax Eclipse C18, 50 x 4.6 mm, 5µm and the mobile phase consists of two Channels A and B.Channel-A:0.1% Trifluoroacetic acid buffer and Channel-B:Acetonitrile. The flow rate is 1.0 ml/min. The column temperature was maintained at 35°C and sample temperature was maintained at 25°C, injection volume 10µLand wavelength fixed at 220nm UV-detection. Retention times of impurities were 10.687 for impurity-A, 8.454 for Impurity-B, 13.018 for Impurity-C, 4.286 for impurity-D, 11.128 for impurity-E and 5.344 for Gatifloxacin.

Preparation of solutions:

Preparation of mobile phase-A:

Transferred 1.0 mL of Trifluoroacetic acid into 1000mLof milli-Q water and mixed well and sonicated to degas.

Preparation of mobile phase-B:

100% acetonitrile.

Preparation of diluent:

Prepared a mixture of 500mL of water and 500 mL of acetonitrile in the ratio of 50:50 (%v/v), filtered through 0.45 µm membrane filter and sonicated to degas.

Preparation of standard solution:

Weighaccurately35.00 mg of Gatifloxacin working standard in to a100 mL volumetric flask, to it added 50 mL of diluent sonicate to dissolved and diluted to volume with diluent and mixed well. Further transferred 1.0mL of this solution into a 100 mL volumetric flask, diluted to volume with diluent and mixed well. Further transferred 1.0 mL of this solution into a 10mL volumetric flask, diluted to volume with diluent and mixed well.

Preparation of test solution:

Weighed accurately and transferred about 35 mg of test sample into a 100 mL volumetric flask, containing 50 mL of diluent sonicated it to dissolved and made up to the volume with diluent.

3. METHOD DEVELOPMENT

3.1. Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

3.2. Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 220 nm from the absorption spectrum.

3.3. Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C18columns could be appropriately used for the separation of related substances for Gatifloxacin.

Poor peak shape and resolution was observed when Inertsil ODS-3V (50mm x 4.6mm, 5µ)There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present. In second attempt made using Zorbax Eclipse C18, 50 x 4.6 mm, 5µm, and gradient mobile phase programmed of Mobile Phase. The resolution of both drug and impurities was achieved.

3.4. Selection of mobile phase

Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of impurities in

Gatifloxacin. A number of column chemistries supplied by different manufacturers and different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Gatifloxacin.

Poor peak shape and resolution was observed mobile phase programmed of Mobile Phase: A phosphate buffer and Mobile Phase: B Acetonitrile. There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present. In second attempt made using mobile phase programmed of Mobile Phase: A 0.1% TFA buffer and Mobile Phase:B Acetonitrile. The resolution of both drug and impurities was achieved. These chromatographic conditions were selected for validation studies.

4. METHOD VALIDATION

4.1. Specificity

Blank interference

Specificity was demonstrated by injected blank solution, standard solution, sample solution, spiked sample and individual impurities and analyzed as per the test method.

Table 1 and Figures 2 to 4 illustrates that the specificity the chromatograms were recorded for blank, sample and spiked sample solutions of Gatifloxacin and its related substances. specificity studies reveal that the peaks are well separated from each other. Therefore the method is selective for the determination of related substances in Gatifloxacin. It was observed that known impurities are not co eluting with each other and main analyte peak. There is no interference of diluent at Gatifloxacin and impurities peaks.

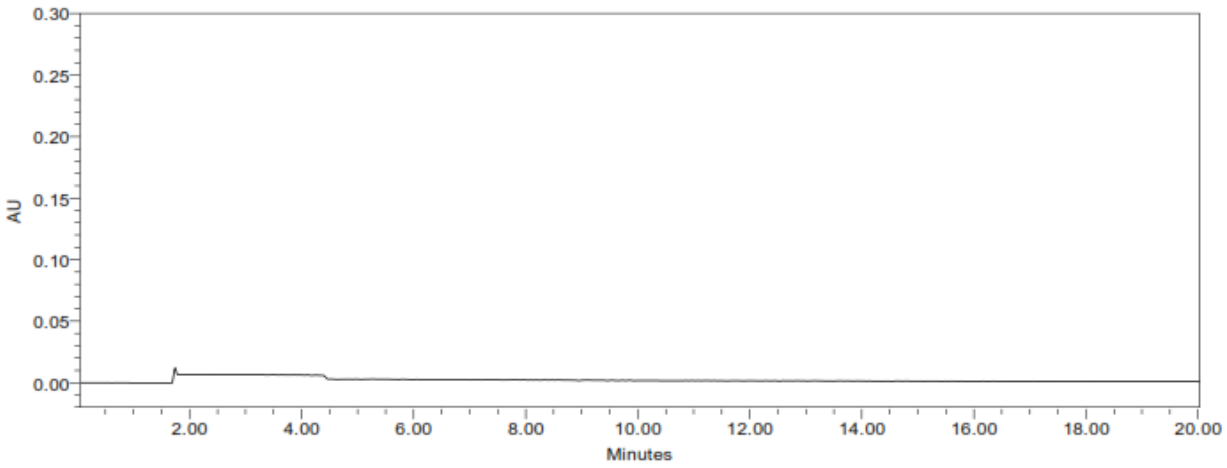

Figure 2. Typical chromatogram of Blank

Slika 2. Tipični hromatogram za prazno

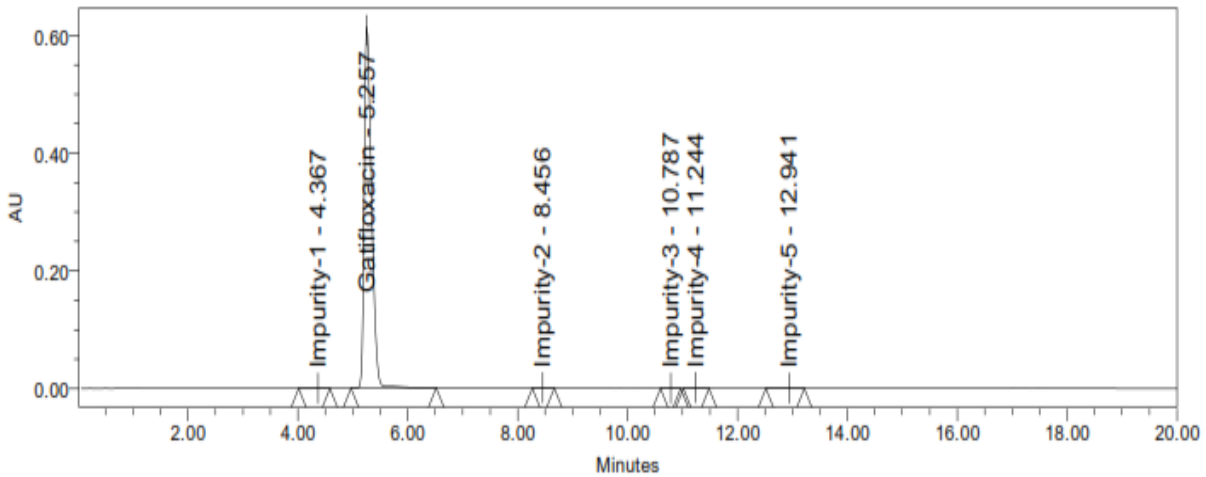

Figure 3. Typical chromatogram as such Sample

Slika 3. Tipičan hromatogram za uzorak

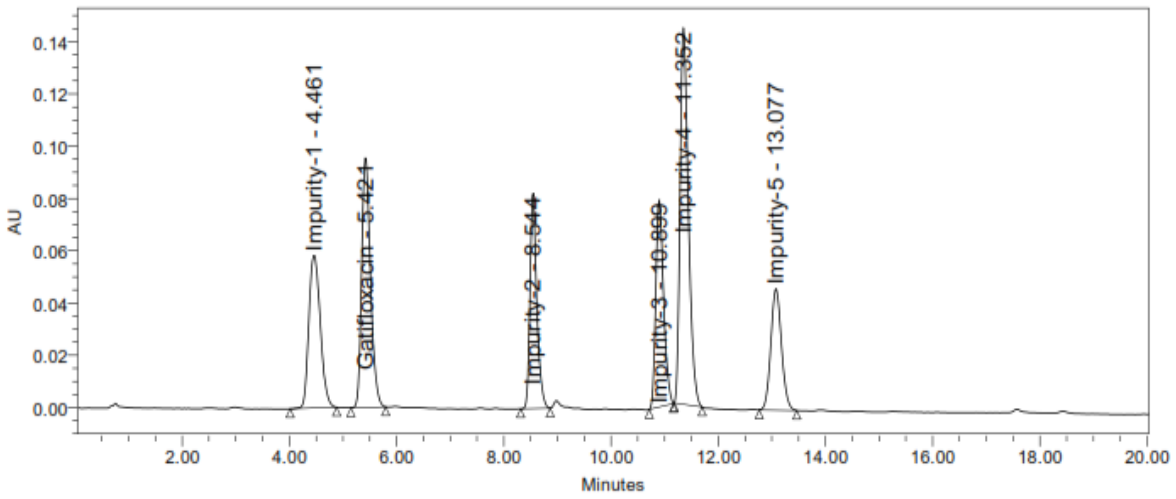

Figure 4. Typical chromatogram Spiked Sample

Slika 4. Tipični hromatogramski šiljasti dijagram za uzorak

Table 1. Impurity interference data (Specificity results)

Tabela 1. Podaci o interferenciji nečistoća (rezultati specifičnosti)

Peak Name	Retention Time	Blank	Placebo
Blank	ND	NA	NA
Impurity-1	4.461	No	No
Impurity-2	8.544	No	No
Impurity-3	10.899	No	No
Impurity-4	11.352	No	No
Impurity-5	13.077	No	No
Gatifloxacin	5.421	No	No

Force degradation

Forced degradation studies were performed to identify potential degradation products that might be formed in Gatifloxacin to elucidate the mechanisms of formation. From those studies it can be seen that there is a significant degradation in solution which exposed to acid, base and water, no significant change was observed. A mild degradation was observed under peroxide conditions. The observations are tabulated below Table 2.

Table 2. Force Degradation results of Gatifloxacin

Tabela 2. Rezultati razgradnje sile gatifloksacina

Name of the Sample	Stress condition	Results in %				
		Imp-01	Imp-02	Imp-03	Imp-04	Imp-05
Control sample	N/A	ND	ND	ND	0.08	0.18
Thermal sample	At 60°C for 2hrs	ND	ND	ND	0.06	0.14
UV exposure sample	2hrs	ND	ND	ND	0.06	0.16
Water hydrolysis sample	Heated at 60°C in H2O for 2Hrs	ND	ND	ND	0.07	0.17
Acid hydrolysis sample	Heated at 60°C in 1.0NHCl for 2Hrs	ND	ND	ND	0.07	0.14
Base hydrolysis sample	Heated at 60°C in 1.0NNaOH for 2Hrs	ND	ND	ND	0.08	0.18
Oxidation sample	Heated at 60°C in 10% H2O2 for 2Hrs	ND	ND	ND	0.38	1.56

The degradation studies indicate that the compound is stable upon exposed to thermal and UV exposed condition. It is concluded from the above observations; the present method is capable to separate all the process and degradation impurities. Therefore the Related compounds by HPLC method is stability indicative.

4.2. Precision

4.2.1. System Precision

Prepared Gatifloxacin(0.1%) and its impurities standard solution (0.15%) and injected six replicates and calculated the %RSD for the peak areas of Gatifloxacin and its impurities. Table 3 and Figure 5 illustrates that the system precision of the method.

Table 3. System Precision data for Gatifloxacin

Tabela 3. Podaci o preciznosti sistema za gatifloksacin

Compound names	Average area	%RSD
Impurity-1	6397	1.4
Impurity-2	11307	0.8
Impurity-3	7332	0.8
Impurity-4	15539	0.4
Impurity-5	10052	0.3
Gatifloxacin	10597	1.3

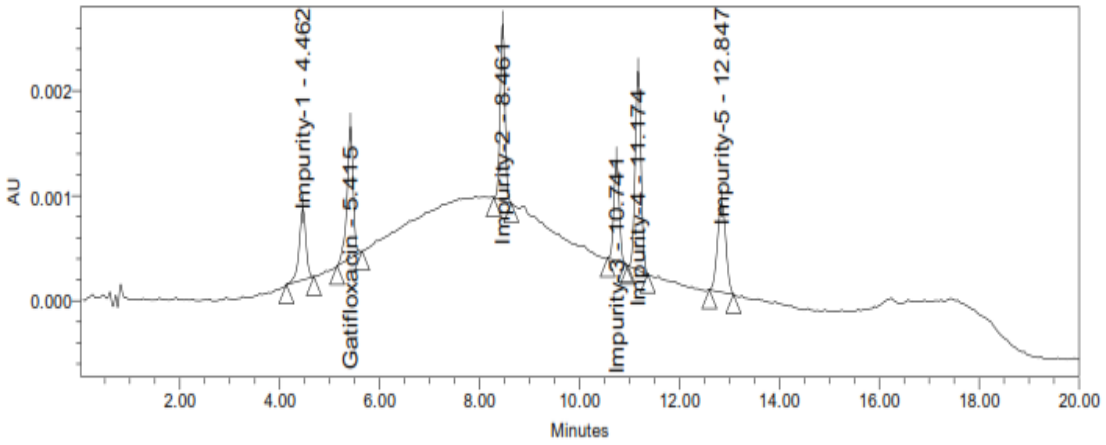

Figure 5. Typical chromatogram standard solution

Slika 5 Tipični hromatogram za standardni rastvor

The % RSD of peak area for Gatifloxacin and its impurities peak from the standard solution observed between 0.3%-1.4% which is below 5.0% indicates that the system gives precise result.

4.2.2. Method Precision

Method precision was demonstrated by prepared six samples by spiking of impurities at specification level and analyzed as per the method (Table 4).

Table 4. Results of method precision

Tabela 4. Rezultati preciznosti metode

S.No.	Sample Details	Impurity (%)				
		Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
1	Prep-1	0.145	0.152	0.157	0.152	0.155
2	Prep-2	0.145	0.152	0.157	0.152	0.152
3	Prep-3	0.145	0.152	0.157	0.152	0.154
4	Prep-4	0.143	0.152	0.156	0.152	0.151
5	Prep-5	0.140	0.151	0.155	0.151	0.158
6	Prep-6	0.143	0.152	0.157	0.152	0.157
Avg.		0.144	0.152	0.157	0.152	0.155
Std. Dev.		0.002	0.000	0.001	0.000	0.003
%RSD		1.38	0.27	0.53	0.27	1.77

Table 4 illustrates that the method precision was demonstrated by prepared six samples by spiking of impurities at specification level and analyzed as per the method. The results six samples were well within the limits. From the above results, it is concluded that method is precise.

4.3. Limit of detection (LOD) & Limit of Quantitation (LOQ)

Limit of detection: The worst found signal to noise ratio for each peak was greater than 3 in each injection. All the peaks were detected in all the three injections.

Limit of Quantitation: The worst found signal to noise ratio for each peak was greater than 10 in each injection. All the peaks were detected in all the six injections.

Table 5 and Figures 6 to 7 illustrates that the LOD and LOQ of the chromatograms were recorded. The limit of quantitation and limit of detection values obtained for each impurity and Gatifloxacin are within the acceptance criteria.

Table 5. LOD&LOQ concentrations and S/N values for Gatifloxacin and impurities

Tabela 5 LOD&LOQ koncentracije i S/N vrednosti za gatifloksacin i nečistoće

Name of the Impurity	Concentration in ppm		Signal to noise ratio value	
	LOD	LOQ	LOD	LOQ
Impurity-1	0.004	0.010	3.01	9.31
Impurity-2	0.004	0.011	4.87	9.26
Impurity-3	0.004	0.014	3.04	9.45
Impurity-4	0.004	0.012	4.87	9.81
Impurity-5	0.004	0.015	3.04	9.45
Gatifloxacin	0.004	0.014	3.02	9.75

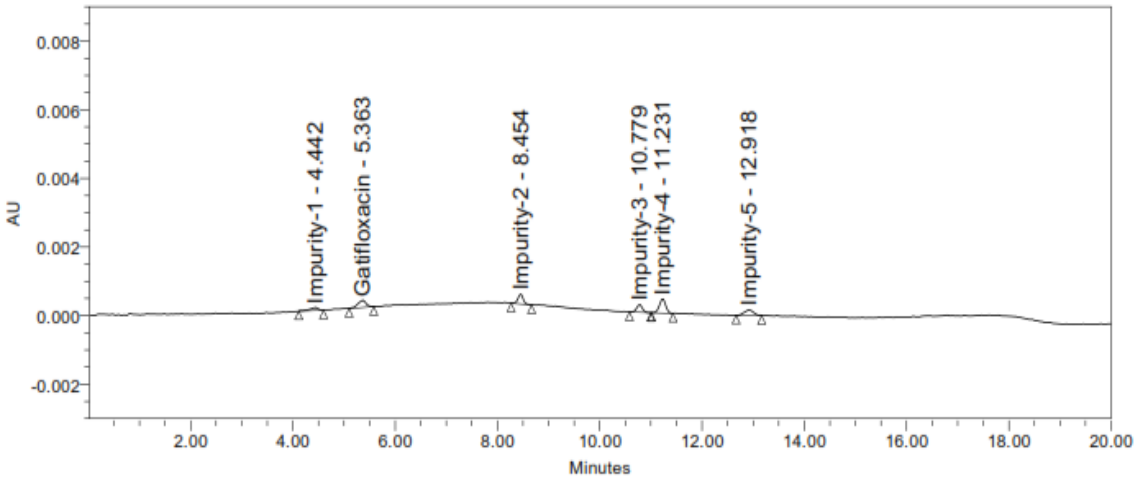

Figure 6. Typical chromatogram LOD solution

Slika 6. Tipični hromatogram za LOD rastvor

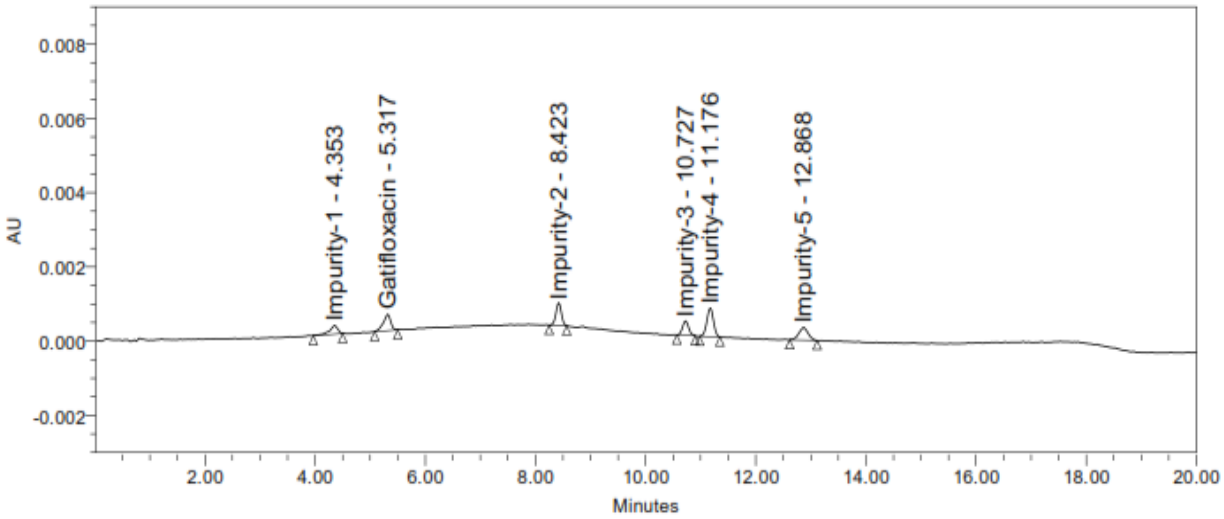

Figure 7. Typical chromatogram LOQ solution

Slika 7. Tipični hromatogram za LOQ rastvor

4.4. Linearity

The linearity of detector response for analytes was demonstrated by preparing solutions over the range of LOQto150% of specification limit with respect to sample concentration. These solutions were injected into the HPLC system and the responses of the same were recorded. A plot of concentration vs. peak area was done. The Coefficient of determination between concentration and response was evaluated.

Table 6 to Table 10 and Figures 8 to 12 illustrates that the linearity results for Gatifloxacin and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99 respectively.

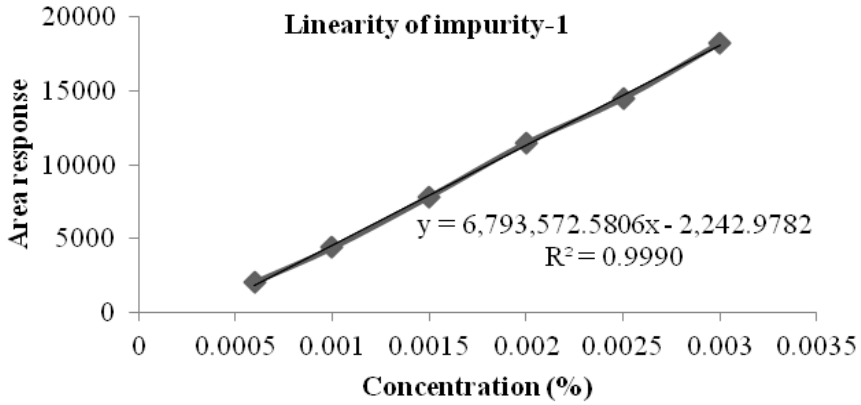

Figure 8. Linearity graph of Impurity-1

Slika 8. Grafik linearnosti nečistoće-1

Table 6. Linearity for Impurity-1

Tabela 6. Linearnost za nečistoću-1

S.No	Levels	Concentration in %	Area response
1	Linearity Level-1	0.0006	4069
2	Linearity Level-2	0.001	6538
3	Linearity Level-3	0.0015	11000
4	Linearity Level-4	0.002	14515
5	Linearity Level-5	0.0025	18297
6	Linearity Level-6	0.003	22655
Square root of Correlation coefficient (r^2)			0.9990
Slope			6793572.5806
Intercept			-2242.9782

Table 7. Linearity for Impurity-2

Tabela 7. Linearnost za nečistoću-2

S.No	Levels	Concentration in %	Area response
1	Linearity Level-1	0.0006	4216
2	Linearity Level-2	0.001	5995
3	Linearity Level-3	0.0015	8710
4	Linearity Level-4	0.002	11307
5	Linearity Level-5	0.0025	14144
6	Linearity Level-6	0.003	16774
Square root of Correlation coefficient (r2)			0.9993
Slope			5288951.6129
Intercept			847.1855

Table 8. Linearity for Impurity-3

Tabela 8. Linearnost za nečistoću-3

S.No.	Levels	Concentration in %	Area response
1	Linearity Level-1	0.0006	3043
2	Linearity Level-2	0.001	4417
3	Linearity Level-3	0.0015	5697
4	Linearity Level-4	0.002	7332
5	Linearity Level-5	0.0025	8678
6	Linearity Level-6	0.003	10442
Square root of Correlation coefficient (r^2)			0.9984
Slope			3023564.5161
Intercept			1259.8694

Table 9. Linearity for Impurity-4

Tabela 9. Linearnost za nečistoću-4

S.No.	Levels	Concentration in %	Area response
1	Linearity Level-1	0.0006	6316
2	Linearity Level-2	0.001	8991
3	Linearity Level-3	0.0015	11993
4	Linearity Level-4	0.002	15539
5	Linearity Level-5	0.0025	18037
6	Linearity Level-6	0.003	21576
Square root of Correlation coefficient (r^2)			0.9986
Slope			6291145.1613
Intercept			2627.6435

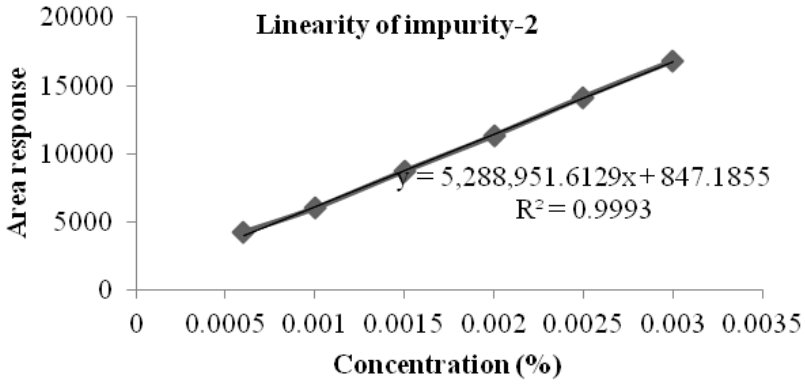

Figure 9. Linearity graph of Impurity-2

Slika 9. Grafik linearnosti nečistoće-2

Table 10. Linearity for Impurity-5

Tabela 10. Linearnost za nečistoću-5

S.No	Levels	Concentration in %	Area response
1	Linearity Level-1	0.0006	3868
2	Linearity Level-2	0.001	5688
3	Linearity Level-3	0.0015	7935
4	Linearity Level-4	0.002	10052
5	Linearity Level-5	0.0025	12790
6	Linearity Level-6	0.003	14768
Square root of Correlation coefficient (r^2)			0.9988
Slope			4584483.8710
Intercept			1084.2452

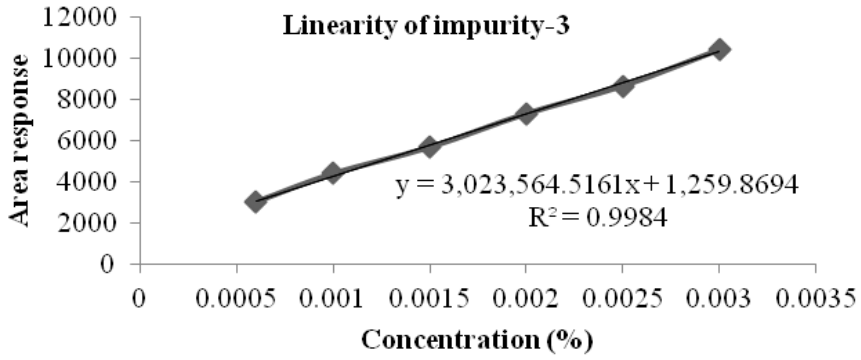

Figure 10. Linearity graph of Impurity-3

Slika 10. Grafik linearnosti nečistoće-3

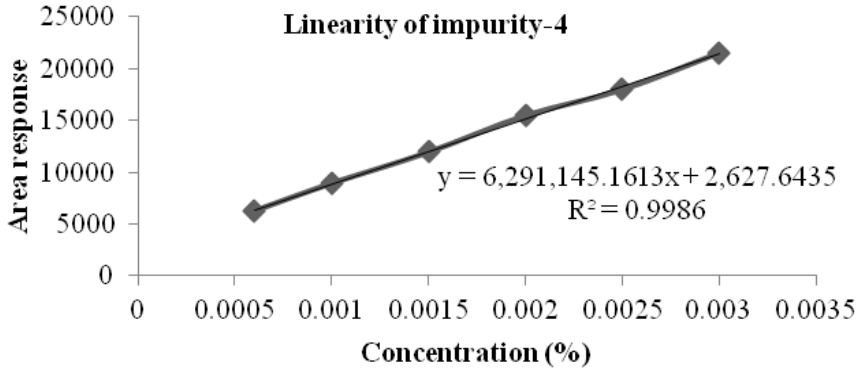

Figure 11. Linearity graph of Impurity-4

Slika 11. Grafik linearnosti nečistoće-4

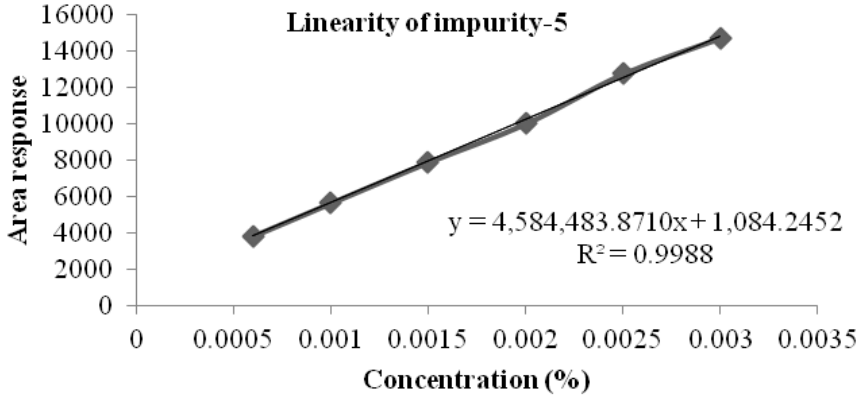

Figure 12. Linearity graph of Impurity-5

Slika 12. Grafik linearnosti nečistoće-5

4.5. Accuracy

Recovery of Gatifloxacin impurities in Gatifloxacin was performed. The sample was taken and varying amounts of Gatifloxacin impurities representing LOQ to 150% of specification level were added to the flasks.

The spiked samples were prepared as per the method and the results are tabulated in Table 11.

Table 11. Accuracy study of Gatifloxacin

Tabela 11. Studija tačnosti gatifloksacina

S.No.	Theoretical (%)	% Mean Recovery				
		Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
1	LOQ	110	93.9	92.9	93.9	92.9
2	50	97.4	97.3	96.4	97.3	96.4
3	100	98.5	99.1	98.2	99.1	98.2
4	150	99.0	100.0	100.6	100.0	100.6

4.6. Solution stability of analytical solutions

Reference solution and spiked test solution prepared, injected different time intervals and calculated the %RSD for the peak area of Gatifloxacin from reference solution, %RSD for the content of impurities from spiked test solution. The reference and test solution stable upto 48 hrs.

5. RESULTS AND DISCUSSION

A simple, economic, accurate and precise HPLC method was successfully developed. The method developed was statistically validated in terms of selectivity, accuracy, linearity, precision, and stability of solution.

For selectivity, the chromatograms were recorded for standard and sample solutions of Gatifloxacin and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore the method is selective for the determination of related substances in Gatifloxacin. There is no interference of diluent at Gatifloxacin and impurities peaks. The elution order and the retention times of impurities and Gatifloxacin obtained from individual standard preparations and mixed standard preparations are comparable.

The limit of detection (LOD) and limit of quantitation (LOQ) for Gatifloxacin standard 0.014 and 0.004, impurity-10.010 and 0.004, impurity-2 0.011 and 0.004, impurity-3 0.014 and 0.004, impurity-4 0.0012 and 0.004 µg/mL and impurity-5 0.015 and 0.004 µg/mL respectively.

he linearity results for Gatifloxacin and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.Calibration curve was plotted and correlation co-efficient for Gatifloxacin and its impurities found to be 0.9990, 0.9993, 0.9984, 0.9986 and 0.9988 respectively.

The accuracy studies were shown as %recovery for Gatifloxacin and its impurities at specification level. The limit of % recovered shown is in the range of 80 and 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

For precision studies six replicate injections were performed. %RSD was determined from the peak areas of Gatifloxacin and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits.

6. CONCLUSION

The new HPLC chromatographic method developed for Gatifloxacin and its related substances are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality during its formulation.

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Conflict of interests

The authors claim that there is no conflict of interest

Abbreviations

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

LOD: Limit of Detection

LOQ: Limit of Quantification

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IZVOD

RAZVOJ I VALIDACIJA RP-HPLC METODE ZA KVANTITATIVNU PROCENU SRODNIH SUPSTANCI U SUPSTANCI LEKA GATIFLOKSACIN

Opisana je analiza poboljšane RP-HPLC metode za odvajanje i kvantifikaciju gatifloksacina i njegovih nečistoća. Uzorci se analiziraju pomoću reverzne faze (RP-HPLC) korišćenjem Zorbak Eclipse C18, 50 x 4,6 mm 5 µm, a mobilna faza se sastoji od dva kanala A i B. Kanal-A: 0,1% pufer trifluorosirćetne kiseline i kanal-B: acetonitril. Brzina protoka je 1,0 ml/min. Temperatura kolone je održavana na 35°C, a temperatura uzorka je održavana na 25°C, zapremina injekcije 10 µLand talasna dužina fiksirana na 220 nm UV-detekcija. Razvijena LC metoda je validirana s obzirom na specifičnost, preciznost, linearnost, robusnost i robusnost. Studija validacije upoređena prema ICH smernicama.

Ključne reči: *Gatifloksacin, određivanje srodnih supstanci, tečna hromatografija*

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