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AQbD Approach- RP-HPLC Method for Optimization, Development and Validation of Garenoxacin Mesylate in Bulk and in Tablets

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ABSTRACT

Garenoxacin Mesylate (GRN) is a quinolone antibacterial agent for the management of bacterial illness. The established method illustrates a development of simple, specific and robust method for the analysis of GRN using a reversed phase high-performance liquid-chromatography method on PrincetonSPHERE ULTIMA C18 column (250 mm x 4.6 mm, 5 µm) with PDA detection was carried out at 280 nm. A seven-factor eight-run Taguchi design was applied to factor screening studies and central composite design with α = 1 was utilized to optimization of experimental parameters of RP-HPLC for obtaining anticipated chromatographic resolution. Risk assessment, examine was executed to understand the basic method parameters. From the risk assessment three independent parameters such as percent acetonitrile content, mobile phase pH and flow rate were selected and study the impact of these parameters on the responses. From the design information the optimized chromatographic conditions comprises of acetonitrile: water in the ratio of 60:40 % *v/v*, pH 3.5 of aqueous phase marked using 0.1 % ortho phosphoric acid, separately. The percent recovery study was executed at three levels, was obeyed in the range of 99 - 101 %. Additional the method was validated as per ICH guidelines.

Keywords: Garenoxacin Mesylate, Analytical Quality by Design (AQbD), Central Composite Design (CCD), method development, validation

INTRODUCTION

Garenoxacin Mesylate (GRN) is 1-Cyclopropyl-8-(difluoromethoxy)-7-[(1R)-1-methyl- 2,3-dihydro-1*H*-isoindol-5- yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid methanesulfonate, chemical structure is shown in **Figure 1** [1].

GRN is utilized in the treatment of certain respiratory tract diseases, Urinary tract infection, otorhinolaryngological infections and Penicillin and fluoroquinolone-resistant Streptococcus pneumonia [2]. A compressive literature survey revealed that, fewer analytical methods *viz* RP-HPLC has been reported for estimation of GRN in pharmaceutical dosage form [3, 4] and in biological samples [5]. Also, Spectrophotometry methods [6 - 8] have been established. Taguchi orthogonal array (TOA) design, which gives an identification of independent factors primary impact on responses in a small number of experimental runs, is being utilized generally in the formulation and development sector because of its robust optimization of process parameters [9-11]. To our comprehension no methods has been found in literature for determination of GRN using analytical quality by design approach. Therefore, the focus of the present research investigation was to establish a simple, accurate, precise and robust RP-HPLC method for analysis of GRN in bulk and in tablets. Further, to study the applications analytical quality by design approach for optimization of chromatographic conditions for the proposed method. Further, studies have been taken to validate established method as per ICH guideline [12].

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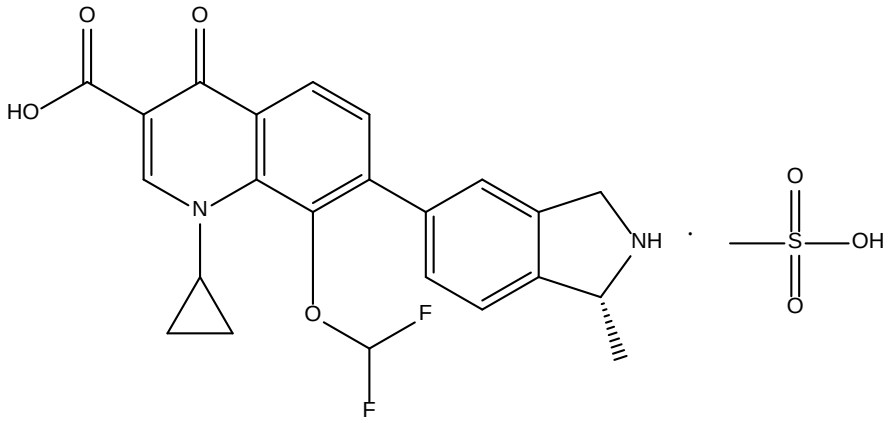

Figure 1. Chemical structure of Garenoxacin Mesylate

CHEMICALS AND REAGENTS

Garenoxacin Mesylate was obtained as a gifts sample from Glenmark Pharmaceuticals Ltd, Ahmadabad, India. HPLC grade Acetonitrile and water was acquired from Merck Mumbai, India.

INSTRUMENTATION

Chromatographic analysis was achieved on Ultra fluid liquid chromatography (UFLC–LC 20 AD), Shimadzu Corporation, Japan. The software implemented for supervising the equipment and processing the data with LC solution Shimadzu Corporation, Japan. SHIMADZU AUX-120 analytical balance was used for weighing Garenoxacin Mesylate. Ultrasonicator; ENERTECH Electronics Pvt. Ltd., India was used for degassing of Stock standard solution and dilutions. Design Expert® (Version: 8.0.4.1), Stat-Ease Inc., Minneapolis, USA software was implemented for statistical determinations.

PREPARATION OF STOCK STANDARD SOLUTION

The stock standard solution was set by dissolving 10 mg of GRN in 100 mL of mobile phase composed of mixture of Acetonitrile: water 60:40 % *v/v* to achieve final concentration of 100 µg/mL.

METHOD DEVELOPMENT AS PER THE EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

In the beginning, the risk assessment plan, Ishikawa fish-bone diagram were constructed utilizing Microsoft Excel to structure the risk operation plan for determining the causes and sub causes affecting the method Critical Analytical Attributes (CAAs). Seven-factor, eight-run Taguchi design was implemented for factor screening studies to know the Critical Method Parameters (CMPs) / Critical Process Parameters (CPPs) which decisively affects the method CAAs (i.e., retention time, theoretical plates and peak tailing) [10, 11]. The design matrix enrolled the considered factors and the decoded translation of their own low and high levels was shown in **Table 1**.

Table 1. Seven-factor eight-run Taguchi design matrix for screening of method variables and process parameters at their respect low and high levels

Run	Mobile phase ratio	Flow rate	Column oven Temperature	Mobile phase pH	Flow	Column	Detector
1	-	+	+	+	+	+	-
2	+	+	-	-	+	-	-
3	-	-	-	-	-	+	-
4	+	-	+	+	-	-	-
5	-	+	+	-	-	-	+
6	+	+	-	+	+	+	+
7	+	-	+	-	+	+	+
8	-	-	-	+	+	-	+

Levels of the factor studied		
	Low (-1)	**High (+1)**
Mobile phase ratio (% *v/v*)	65:35	55:45
Flow rate (mL/min)	0.8	1.2
Column oven temperature (°C)	25	35
Mobile phase pH	3	3.5
Flow type	Isocratic	Gradient
Column type	Qualisil	Princeton
Detector	UV	PDA

Table 2. Design Matrix as per CCD for Optimization of LC Method

Run	Mobile phase ratio	Flow rate	pH
1	0	-α	0
2	1	1	1
3	1	1	-1
4	0	0	-α
5	0	0	0
6	-α	0	0
7	0	0	0
8	0	0	0
9	0	α	0
10	0	0	0
11	0	0	0
12	A	0	0
13	-1	1	1
14	0	0	α
15	-1	1	-1
16	0	0	0
17	1	-1	-1
18	-1	-1	1
19	-1	-1	-1
20	1	-1	1

Factors	**-α**	**-1**	**0**	**+1**	**+α**
Mobile phase Ratio (%v/v)	Acetonitrile: water (68:32)	Acetonitrile: water (65:35)	Acetonitrile: water (60:40)	Acetonitrile: water (55:45)	Acetonitrile: water (52:48)
Flow rate	0.66	0.80	1.0	1.2	1.33
Mobile phase pH	2.82	3	3.25	3.5	3.67

The sum of eight design runs was attained and the experimental design runs were analyzed for the influence of study factors on the CAAs. The preliminary risk assessment and factor screening studies, selection of the CMPs absolutely affecting the method performance was board upon for further method optimization. CCD with α = 1 was used for optimization of selected CMPs, specifically, the mobile phase ratio, flow rate and mobile phase pH studied at four levels, that is, low, high, particularly high and extremely low. **Table 2** summarized design matrix consisting of 20 experimental runs as per CCD, as well as a total of fourteen experimental runs together with six runs of the center point (0, 0). A standard concentration of 10 µg/mL was used for all the experimental runs that were analyzed for CAAs, prominently for retention time, tailing factor and theoretical plates.

All the results were assessed using statistical software Design Expert version 8.0.4.1 (Stat-Ease Inc, Minneapolis, MN). The design was analyzed by choosing the second order polynomial model for estimation of main effect(s) along with interaction effect(s). The linear polynomial equations generated from ANOVA are in the form, is show below.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 - b_{13}x_1x_3b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 - b_{33}x_3^2 \quad (1)$$

where, y is the measured response (dependent variable) associated with the each factor level combination; b_0 represents the polynomial equation intercept representing average arithmetic mean of all quantitative outcomes of runs and $b_1 - b_{33}$ is regression coefficients computed from the observed experimental values of y, y_1 and y_2, x_1, x_2, x_3 and x_3 represent the coded levels of independent variables.

VALIDATION OF PROPOSED METHOD

The established method was validated for linearity, specificity, accuracy, precision and limit of detection (LOD), limit of quantification (LOQ) according to the guidelines of ICH 2005.

System Suitability

For system suitability studies, six concentrations of 10 µg/mL of Garenoxacin Mesylate were injected and analyzed by RP-HPLC as per ICH guidelines. From these replicates injections, the appreciation criteria for theoretical plates, tailing factor and capacity factor were taken into consideration.

Linearity

The linearity for Garenoxacin Mesylate was plotted the range of 2 - 12 µg/mL. The analysis was performed in six times for each concentration. The calibration curve was studied as peak areas *versus* drug concentration. The linearity curve was interpreted by linear least square regression analysis.

Precision

Precision was reported in terms of repeatability (intra-day variation) and intermediate (inter-day variation) precision, according to the ICH guidelines. Inter-day and intra-day evalution were recorded by determination of six replicates of three concentrations (4, 6 and 8 µg/mL) for a period of three successive days.

Accuracy

The accuracy of the proposed method was assessed in terms of percentage recovery studies at three different levels 80 %, 100 %, and 120 %. The % recovery study was performed by adding known amount of drug standard solution to the pre-analyzed sample and it was re-analyzed by proposed HPLC method.

Sensitivity

The sensitivity evaluation of proposed methods were anticipated in terms of the Limit of Limit of Detection (LOD) and Quantification (LOQ). The LOD is the smallest amount of the drug which can be detected by the proposed method and LOQ is smallest amount of drug which can be quantified by the proposed method. The LOD and LOQ were calculated using equation LOD = 3.3 × N / B and LOQ = 10 × N / B, where, 'N' is the standard deviation of the results of the drugs (n = 3), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve.

Specificity and Selectivity

The analytes should have no hindrance from other unrelated components and be well resolved from them. Specificity is a procedure to detect quantitatively the analyte in the presence of components that may be likely to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be anticipated to be present in the sample matrix.

Ruggedness

The ruggedness studies were performed by analyzing appropriate concentration of 6 µg/mL by two different analysts without making no change in operational and environmental conditions.

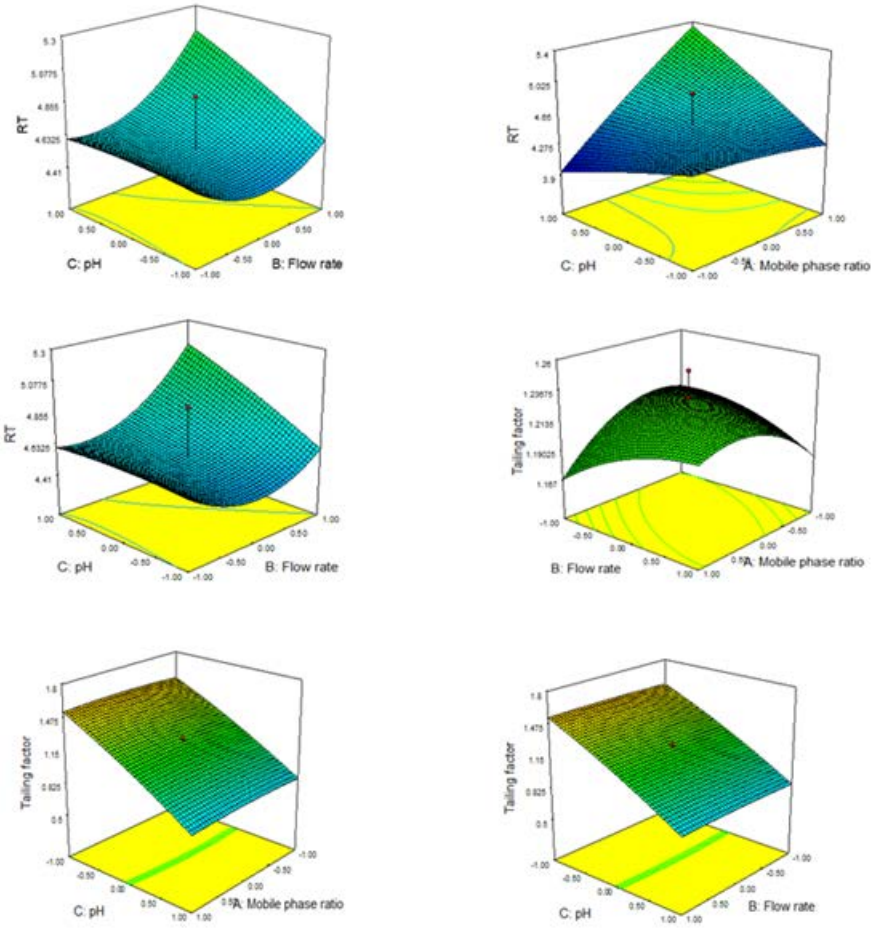

Figure 2. 3 - D plots for the CAAs, namely (A) Retention time, (B) Tailing factor and (C) Theoretical plates

RESULTS AND DISCUSSION

Optimization of Data Analysis and Statistical Analysis

The optimization, data analysis was carried out by selecting the second-order quadratic polynomial model for detecting of retention time, tailing factor and theoretical plates with design expert software version 8.0.4.1as depicted in equations (2) – (4) as follows:

$$Y_1 = 4.57 + 0.30x_1 + 0.13x_2 + 0.13x_3 + 0.32x_1x_2 + 0.40x_1x_3 + 0.13x_2x_3 - 0.036x_1^2 + 0.19x_2^2 - 0.022x_3^2 \quad (2)$$

$$Y_2 = 4.57 + 0.30x_1 + 0.13x_2 + 0.13x_3 + 0.32x_1x_2 + 0.40x_1x_3 + 0.13x_2x_3 - 0.036x_1^2 + 0.19x_2^2 - 0.022x_3^2 \quad (3)$$

$$Y_3 = 4.57 + 0.30x_1 + 0.13x_2 + 0.13x_3 + 0.32x_1x_2 + 0.40x_1x_3 + 0.13x_2x_3 - 0.036x_1^2 + 0.19x_2^2 - 0.022x_3^2 \quad (4)$$

where, Y_1 (Retention Time), Y_2 (Tailing factor) and Y_3 (Theoretical plates) are responses; x_1 (Mobile phase ratio), x_2 (Flow rate) and x_3 (pH of aq. phase) were the factors. The 3-D response surface plot observed for CAA i.e. retention time was shown in **Figure 2**.

The figure depicts negative effect of acetonitrile content on the retention time (Rt); while flow rate and pH of aq. phase has no effect on retention time. Lower pH of aq. Phase lead to more peak tailing, while tailing factor remained unaffected by flow rate and mobile phase ratio. Mobile phase ratio had negative effect on theoretical plates, while pH of aq. phase and flow rate found to affect it as before. Final optimum solution was found out with application of numerical optimization as presented in **Figure 3**.

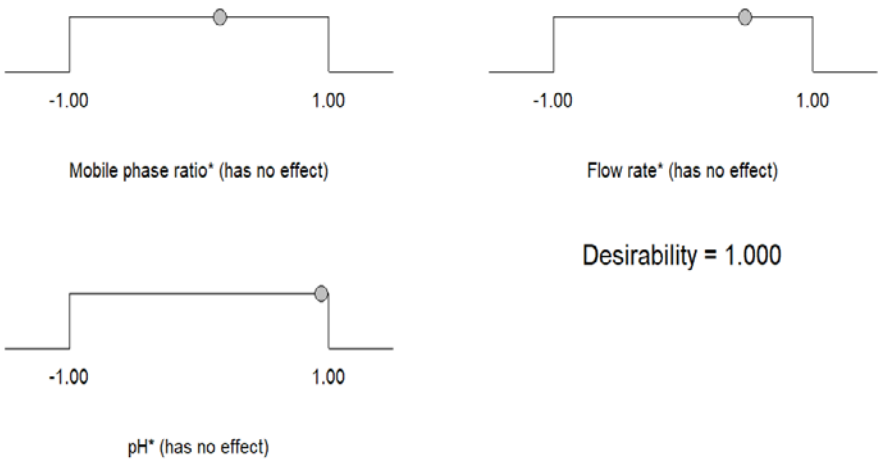

Figure 3. Final Optimum Solution for the Garenoxacin Mesylate

Table 3. Statistical Analysis using ANOVA

Responses	SS	DF	MS	Std. dev	C. V. %	F- value	P- value	Results
Rt	4.54	9	0.50	0.31	6.70	5.17	0.0085	Significant
TF	1.80	9	0.20	0.05	4.28	78.55	0.0001	Significant
TP	7.447E+005	9	82745.86	90.81	4.67	8.31	0.0014	Significant

Table 4. Optimized chromatographic conditions

Chromatographic Mode	Chromatographic Conditions
HPLC System	UFLC-LC 20 AD (Shimadzu Corporation, Japan)
Detector	SPD-M 20A (Diode array detector)
Column	PrincetonSPHERE ULTIMA C18 (250 mm × 4.6 mm, 5µm)
Mobile phase	Acetonitrile: water (60: 40 % *v/v*)
Detection wavelength	280 nm
Flow rate	1 mL/min
Injection Volume	20 µL

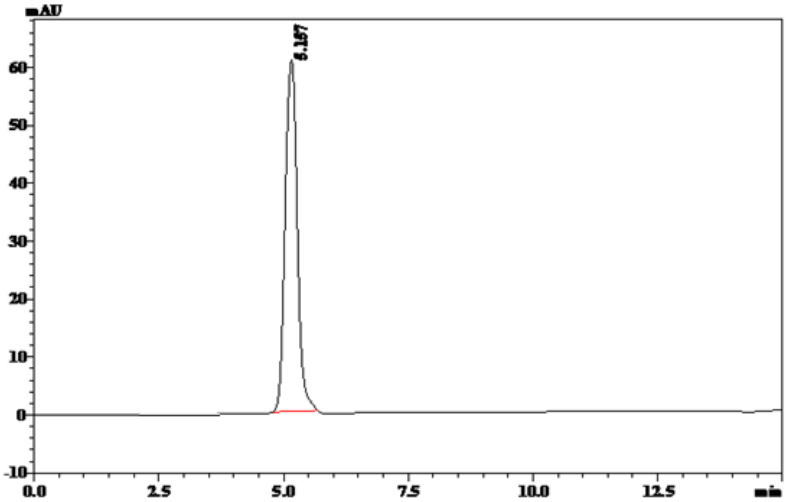

Figure 4. HPLC Chromatogram for Garenoxacin Mesylate

The optimized solution resulted into mobile phase composition containing a mixture of acetonitrile and water 60:40 (0.1 % v/v, Ortho Phosphoric acid), flow rate 1.0 mL/min and mobile phase pH of 3.5 with a value for desirability equal to 1.0. The results of ANOVA are as shown in **Table 3**. The optimized chromatographic conditions are represented in **Table 4**. The **Figure 4** illustrates the HPLC Chromatogram of GRN.

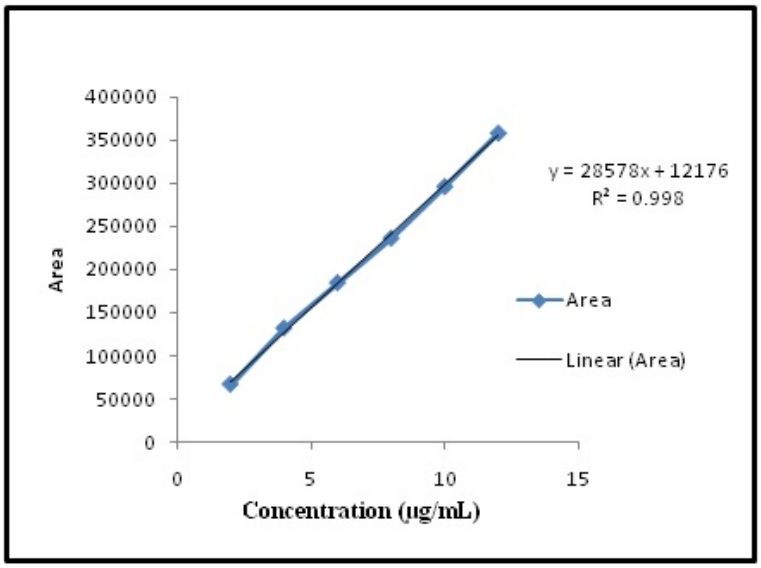

Figure 5. Calibration curve for Garenoxacin Mesylate

Table 5. Precision studies

Standard Concentration [µg/mL]	Amount Found [µg/mL]	% Amount found [µg/mL] [n=3]	% RSD
Intra-day Precision			
4	3.98	99.71	0.36
6	5.94	99.01	0.43
8	7.94	99.34	0.85
Inter-day Precision			
4	4.02	100.52	0.37
6	5.99	99.98	0.47
8	7.96	99.50	1.20

n- number of determinations

METHOD VALIDATION

System Suitability

The results of system suitability testing in the forms of values for the retention time as 5.15 min; theoretical plates as 2257.0; tailing factor 1.045; capacity factor 1.063 and % RSD calculated for six replicate injections were found to be in acceptable range.

Linearity

The standard calibration curve was plotted in concentration range of 2 to 12 µg/mL. The obeyed linearity range has shown adequate coefficient regression value ($r^2 = 0.998$). The linear regression equation was found with a form y = 28578x + 12176. The calibration curve for the linearity studies is as portrayed in **Figure 5**.

Precision

Intra-day and inter-day precision studies were executed leading to findings that the obtained % RSD values were less than 2.0. The results are as depicted in **Table 5**.

Accuracy

The accuracy of method was ascertained by the standard addition method by adding drug at three different concentration levels. The percent recovery was found to be in the range of 99 - 101 % as shown in **Table 6**. The

Table 6. Accuracy studies

Drug	Initial Amount [µg/mL]	Excess drug added to the analyte [%]	Total amount found ± S.D. [g/mL]	Recovery [%] [n=3]	%RSD [n = 3]
GRN	5	80	9.01 ± 0.02	100.14	0.71
	5	100	9.98 ± 0.04	99.44	1.23
	5	120	11.25 ± 0.04	101.49	0.88

n- number of determinations

Table 7. Summary of the developed RP-HPLC method

Parameter	RP-HPLC
Linearity range [µg/mL]	2 – 12
Correlation coefficient	0.998
Analysis of tablet formulation [% amount Recovered]	99.09
Detection limit [µg/mL]	0.024
Quantification limit [µg/mL]	0.073
% Recovery	99 - 101
Ruggedness [% RSD, n=6]	
Analyst- I	0.58
Analyst- II	0.95
Precision [% RSD]	
Intra-day [n = 6]	0.36 - 0.85
Inter-day [n = 6]	0.37 - 1.20
Repeatability	

n- number of determinations

results demonstrated that the created technique is exact and might be utilized as a part of the routine exploratory investigation of GRN.

Sensitivity

The minimum concentration at which an analyte was detected reliably (LOD) and quantified (LOQ) was found to be **0.024** and **0.073 µg** respectively.

Ruggedness

An appropriate concentration of 6.0 µg/mL sample solution was analyzed by two different analysts utilizing comparative operational and atmospheric conditions. Peak area was assessed for the same concentration solutions, six times; the result was shown in **Table 7.**

Selective

The method was quite selective as there were no other obtrusive peaks around the retention time of GRN; also the baseline was devoid of any noise.

ANALYSIS OF TABLET FORMULATION

GRN tablets (label claim 200 mg) were procured from local market**;** twenty were accurately weighed and grounded into fine powder. A quantity of powder drug equivalent to 200 mg of Garenoxacin was transferred into 100 mL volumetric flask which previously contained 50 mL of methanol, sonicated for 10 min and the volume of the solution was made up to the mark using same solvent. The appearing solution was filtered through a 0.45 µm filter (Millifilter, Milford, MA, USA). From the filtrate, a fixed volume of solution was transferred using calibrated pipettes into 10 mL volumetric flasks and the volume was made up to the mark with mobile phase to achieve concentrations of 5 µg/mL and resultant solution was analyzed. The concentration was determined using linearity equation.

CONCLUSION

A simple, robust, specific, precise and sensitive RP-HPLC analytical method using Analytical quality by design approach has been developed for quantitative determination of GRN in bulk and in tablets. Application of

Analytical Quality by design approach and experimental design permitted assessing the chosen factors jointly, including interaction between factors, by method of a rational approach so as to come to the optimum chromatographic conditions. The 3D response surface graphs show that the percent acetonitrile content in mobile phase was found to be the most crucial factor for retention time of GRN. The acceptable percent recovery in formulation marked that the excipients present in the formulation have no hindrance in the determination. The results of system suitability, linearity, precision, accuracy, specificity and LOD, LOQ confirmed that developed RP-HPLC analytical method using AQbD approach can be implemented for regular analysis of GRN formulations.

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