Original Article

Application of Design of Experiment Based Innovative Approach in Method Development and Validation of RP-HPLC for Estimation of Azilsartan in Bulk and Pharmaceutical Tablet Dosage Form

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ABSTRACT

Aim: Azilsartan is an angiotensin II receptor blocker used in the treatment of hypertension. Using the DoE based approach in reversed-phase high-performance chromatography method was developed and validated as per ICH guidelines. **Materials and Methods:** The separation of Azilsartan using the Qualisil 5 BDS C_{18} column (250 x 4.6mm, particle size 5μ) in low-pressure gradient mode with photodiode array detector at 249nm. For optimization of chromatographic conditions for Azilsartan from its formulation with less number of experimental trials using Box-Behnken design. The critical quality attributes that are acetonitrile concentration in the mobile phase, pH of the aqueous phase and flow rate parameters were used to construct a mathematical model and study the effects of these independent factors on response such as retention time, tailing factor and theoretical plates. Analysis of Variance (ANOVA) confirmed that the three factors were significant its p-value found less than 0.05. **Results:** Optimized experimental conditions obtained by the DoE approach for proposed work consists of water and acetonitrile (75:25% v/v), pH 5.0 adjusted with orthophosphoric acid as a mobile phase at a flow rate 1ml/min with a retention time was found to be 3.516min. The developed method was validated as per ICH guidelines. An accuracy study was performed at three different levels and was found in the range of 98.94-100.46%. **Conclusion:** The method was found simple and rapid with good specificity and robustness.

Key words: Azilsartan, ANOVA, Box-Behnken design, DoE approach, Robustness, Validation.

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INTRODUCTION

Azilsartan is a selective AT1 subtype angiotensin II receptor antagonist and peptide hormone used as an antihypertensive drug. The mechanism of action is to lower blood pressure by inhibition of vasopressor hormone Angiotensin II that causes vasoconstriction. It increases blood pressure and aldosterone release.[1-3] Apart from the role of the RAS in cardiac and renal treatment, it has been shown to meet the trademarks of the cancer.[4] In recent studies it plays an important role in different cancer cells and tissues such as liver, lung, breast and pancreas.[5] During absorption in the gastrointestinal tract, Azilsartan medoxomil is hydrolyzed to Azilsartan i.e. the active metabolite. After oral administration it is not detected in plasma. The Azilsartan medoxomil dose values are 40mg and 80mg once in a day. The bioavailability of Azilsartan is approximately 60% and reached within 1.5 to 3 hr. Azilsartan is not

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dialyzable. The 80mg dose is recommended in adults taken by orally once daily.[6,7]

Chemically, Azilsartan is ((5-methyl-2-oxo-1,3-dioxol-4yl) methyl 2-ethoxy1-((2'-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl4-yl) methyl)-1H-benzimidazole-7carboxylate).[3] Azilsartan is crystalline powder in nature having off-white color and soluble in methanol and partially soluble in acetonitrile and insoluble in water. The literature review on the availability of analytical methods for Azilsartan revealed a UV spectroscopic method,[7] few ultra-performance liquid chromatographic method and LC-MS methods for the quantification of Azilsartan in bulk drug, plasma[8,9] and combination with other drugs[10,11] and stability-indicating high-performance thin layer chromatographic (HPTLC) method.[12] In the literature survey, many chromatographic analytical methods were developed having several drawbacks include high buffer concentration leads to column damage, time-consuming and tedious. It needs to develop a method that resolves the problems of the previous methods.[13] In this experiment we are developing a new method by using the DoE approach. The chemical structure is presented in Figure 1.

The concept of quality by design was coined Joseph Juran a quality expert. According to him, quality could be planned to minimize the most quality crises and problems in the first place. QbD encompasses designing and developing formulations and manufacturing processes that ensure predefined product specifications. An understanding of how product and process variables influence product quality are required for QbD. In addition to this new concept being considered by FDA in its cGMP initiative, three important guidance documents were published as part of the International Conference on Harmonization (ICH) guidelines: Q8 (Pharmaceutical Development) and Q9 (Quality Risk Management). Q10 (Pharmaceutical Quality System) also describes a model for an effective quality management system for the pharmaceutical industry. Recently in the literature, the application of QbD principles to pharmaceutical development and manufacturing has added a lot of interest. QbD is planned by pharmaceutical development, quality risk management and pharmaceutics quality system of ICH guidelines.[14-17]

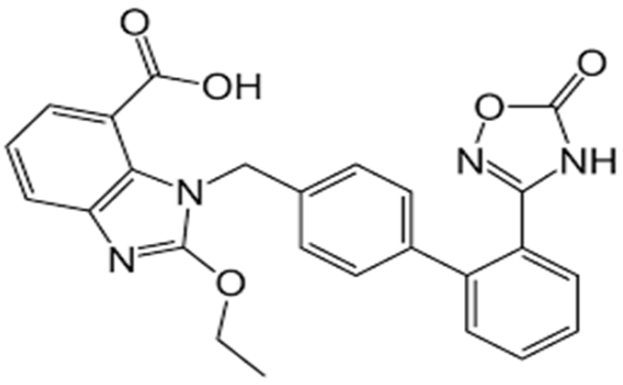

Figure 1: Structure of Azilsartan.

The process of analytical method development using QbD included the first target product profile which defines the outlooks in the final step of method optimization called quality target product profile (QTPP) and also known as an analytical target profile (ATP).[18] The second phase based on system suitability results gives performance criteria for the chromatographic method is developed. The third phase consists of a systematic process for the assessment, control, communication and review of risks to the quality of drug products across the product lifecycle. An assessment of risks. Risk assessment helps to increase the quality of the method or process. It is also a determining factor for the effect of the input variable on the method. From risk assessment can identify critical attributes that are going to affect final results. An Ishikawa or fishbone diagram is used to categorize all potential variables such as material, mobile phase, instrumental parameters, column characteristics and sample preparation.[19] The fourth phase consists of setting chromatographic conditions and the availability of material known as method design. After method development, the software is used to generate data without actual experimentation.[20] The fifth phase includes input material controls, process controls and, monitoring the design space around individual or multiple unit operations and/or final product specifications used to ensure consistent quality. The final phase consists of continuous improvement throughout the process to gain more knowledge and improve method quality.[21]

To estimate Azilsartan in pharmaceutical oral dosage form and biological samples, several analytical methods have been developed. The ICH (International Conference of Harmonization) Q1A(R2) guideline suggests that stress studies should be carried out on a drug to establish stability.[22]

In the present research, the design of experiment approach used to develop, optimized and validated according to ICH guidelines. Hence a new, precise, accurate and robust method was developed for Azilsartan in the pharmaceutical oral dosage form.

Chemical Name: ((5-methyl-2-oxo-1,3-dioxol-4yl) methyl 2-ethoxy1-((2'-(5-oxo-4,5-

dihydro-1,2,4-oxadiazol-3-yl) biphenyl4-yl) methyl)-1H-benzimidazole-7-carboxylate)

Molecular Formula: $C_{25}H_{20}N_4O_5$

Molecular Mass: 456.4 g/mol

Description: an off-white crystalline powder

Category: Antihypertensive

Dose: 40mg, 80mg

Solubility: freely soluble in methanol, slightly soluble in acetonitrile, insoluble in water

Melting Point: 212-214°C

MATERIALS AND METHODS

Instrumentation

A quantitative HPLC system (Shimadzu Corporation, SPD-M20, Japan) equipped with LC solution software was used for LC studies, the detector was a photodiode array detector having a light source of deuterium (D2) and tungsten (W) lamp with a wavelength range of 190-800nm. It had an on-line degasser containing the binary pump and sample injector with a 20 µL loop. Separation studies were carried out using C_{18} column Qualisil 5 BDS-C_{18} (250mm 4mm i.d., 5µm particle size) (Netherlands). pH meter (Labman scientific instrument Pvt. Ltd., Chennai, India) was used to adjust the pH of the mobile phase and other solutions used during the study. Other instruments also used during the study were, Analytical balance (Contech instrument Ltd., Pune, India), Sonicator (Citizen digital ultra sonicator) and suction pump (rocker 300A vacuum filtration system).

Materials

The working standards were a kind gift from Aurobindo pharma Pvt limited, Hyderabad, India. HPLC-grade acetonitrile and water were obtained from Merck Chemicals. The tablet Myotan® with label claim 40mg of Azilsartan from Synokem Pharmaceuticals LTD.

Mobile Phase Composition

In the current research doubled distilled water adjusted to pH 5 by orthophosphoric acid and acetonitrile.

Standard solution preparation

The working standard of 10mg Azilsartan was taken in 10 ml volumetric flask and acetonitrile was added followed by filling to the mark to obtain 1000µg/ml. Transfer 1ml from resulting solution to 10ml volumetric flask and volume was made up with acetonitrile to form 100µg/ml stock standard solution. The 20µg/ml was used for the optimization of the method.

Sample solution preparation

For assay preparation, 20 tablets (Myotan® 40mg) were ground and tablet powder equivalent to 100mg of Azilsartan was taken in a 100ml volumetric flask and diluted with acetonitrile. The solution was filtered through a 0.45µ filter. From the filtrate, an appropriate volume of solution was diluted to get a final concentration of 20µg/ml using acetonitrile.

Preliminary studies for method development

The preliminary studies for method development were conducted using Shimadzu LC20AD high-performance liquid chromatography system (HPLC) with PDA using Lab solutions software. A Qualisil 5 BDS C_{18} column (250 x 4.6mm, particle size 5µ) maintained at ambient temperature was used for the chromatographic separation. Before applying the DoE approach, the chromatographic condition was a low-pressure gradient developed using water (pH adjusted to 5 using orthophosphoric acid) and acetonitrile (80:20 v/v). The mobile phase was degassed and filtered through membrane filter 0.45µ. The flow rate of 0.8ml/min and an injection volume of 20 µL was used. The run time for the analysis was 10 mins. Using a PDA detector, the detection was performed at 249 nm.

Analytical Target Profile (ATP) and Method Qualification

Various components of ATP include characteristics like target drug, quality attributes for chromatographic method, sample preparation, instrument parameters and method application. Hence it begins with goal determination. This attention was given to the product and process understanding. The robust, accurate, precise and USP tailing less than 1.2 number of theoretical plates greater than 2000 and short analysis time i.e. less than 10min was the method intended for the development of Azilsartan.[23,24]

Once the method was designed, the following step arises method qualification this was to confirm that method was carried out as intended. It was classified in design qualification, installation qualification, operational qualification and performance qualification.

Risk assessment in method development

A risk assessment of the drug substance attributes was performed to evaluate the impact that each attribute could have on the drug substance's CQAs. Factors were organized hierarchically using an Ishikawa or "fishbone" diagram also called as cause and effect diagram it was used as a primary tool in the process of risk analysis. It is

simply defined as a diagram that shows the causes of an event. The fishbone diagram was used to assess critical effects caused by material, mobile phase, instrumental parameters, sample characteristics and preparation and column characteristics.[25,26] Another tool for risk assessment conducted FMEA model was also used to found potential risk factors on the process and quality of the product. FMEA was identified by the risk priority number (RPN). The responsibility of risk was carried as severity (S), probability (P), detectability (D).[27,28] It was shown in Figure 2.

Statistical Software

Design Expert (Version 12), Stat-Ease Inc. Minneapolis, MN, USA statistical computer software was operated for method optimization. The results of validation parameters were calculated by Microsoft Excel 2016 software.

Preliminary screening of critical attributes factor

The screening method for the design of experiment was used to identify the critical attribute factors (CQA). C_8 and C_{18} column selection, the concentration of aqueous and organic mobile phase, pH adjustment of the mobile phase, detection of wavelength, the temperature of column, isocratic/gradient flow of mobile phase, flow rate and volume of injection were used for the risk assessment. The process method parameters were initially investigated viz. the number of theoretical plates, retention time and tailing factor. In this experiment, the critical quality attributes were pH of the mobile phase, flow rate and acetonitrile concentration to develop a chromatographic analytical method by using DoE.

RESULTS AND DISCUSSION

Box-Behnken design used for method optimization

Several methods of RP-HPLC for estimation of Azilsartan were developed containing water, potassium dihydrogen phosphate ammonium acetate, to attain good separation of the drug but they are not cost-effective in case of solvent quantity used for regular analysis of Azilsartan in its bulk and pharmaceutical dosage form.[29,30] In the present study, the 3^3 level and factor combinations of the Box-Behnken design type used with response surface methodology and the experiments were planned and conducted. The optimization was done by applying three levels of factors as reported in Table 1. Based on preliminary method development trials the levels were identified and used in statistical software to determine runs. The Box-Behnken design was selected for this study and it requires 13 runs for modeling a response surface. There were three independent factors, i.e. the pH of the mobile phase (X_1), flow rate (X_2), the concentration of solvent in the mobile phase (X_3) on the tailing factor(Y_1), retention time(Y_2), theoretical plates(Y_3).

The quadratic model and regression analysis applied for the USP tailing factor, retention time and theoretical plates of the peak. Regression analysis and p-values obtained from the software-generated reports were given in Table 2. To study the effect of the factors on the responses Analysis of variance (ANOVA) was performed. *p*-values provide the model is 'statistically significant' it is $p < 0.05$. It was shown in Table 3. The remaining confirmation of the method was completed using the concept of 'Desirability' by software analysis. Figure 6.

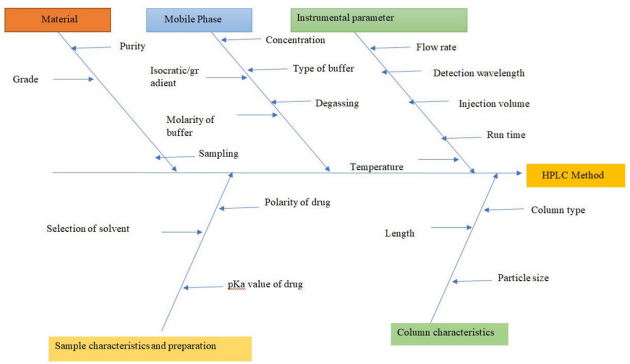

Figure 2: Fishbone diagram for HPLC method development.

Response Surface Methodology by DoE

To recognize the interactions between the variables such as pH of the mobile phase, flow rate and acetonitrile concentration, the response surface diagrams (3D graphs and counter plots) were drawn for the whole experimental design.

Quadratic Model Equations

Tailing factor (Y_1) $=+1.10-0.0700A-0.0613B-0.2438C+0.0950AB+0.1100AC+0.0325BC+0.0938A^2+0.0663B^2+0.0712C^2$

Retention time (Y_2) $=+4.47+0.0841A-1.07B+0.1813C+0.0360AB-1.1447AC-0.0607BC-0.1022A^2+0.1973B^2-0.1790C^2$

Table 1: Experimental plan of Box-Behnken design showing factors and levels.

Code	Factors	Low	Medium	High
X_1	pH	4.5	5	5.5
X_2	Flow rate (ml/min)	0.6	0.8	1
X_3	ACN concentration (%)	15	20	25

Table 2: Design matrix of BBD constructed using critical factors with selected responses.

Sr. No.	pH	Flow rate (ml/min)	ACN conc. (%)	Tailing Factor	Retention time (min)	Theoretical plates
1	4.5	0.8	15	1.65	3.598	4678
2	5.5	0.8	15	1.33	4.162	4941
3	5.5	0.6	20	1.13	5.743	3065
4	4.5	0.8	25	0.98	4.505	5321
5	5.5	1	20	1.21	3.449	4098
6	5	1	25	0.94	3.516	5902
7	5	0.6	15	1.6	5.339	4985
8	5.5	0.8	25	1.1	4.49	4951
9	5	0.6	25	1.01	5.568	3258
10	4.5	1	20	1.2	3.315	3985
11	5	0.8	20	1.1	4.47	5643
12	4.5	0.6	20	1.5	5.753	3179
13	5	1	15	1.4	3.53	4143

Table 3: ANOVA results for response Y1 (Tailing factor), Y2 (Retention time), Y3 (Theoretical plates) obtained from experimental design space

	Source	Sum of squares	Df	Mean square	F value	p-value	
Response Y1 (tailing factor)	Quadratic model	0.6569	9	0.073	34.62	0.007	Significant
	X1	0.0392	1	0.0392	18.59	0.0230	
	X2	0.0300	1	0.0300	14.24	0.0326	
	X3	0.4753	1	0.4753	225.44	0.0006	
Response Y2 (retention time)	Quadratic model	9.97	9	1.11	13.43	0.0278	Significant
	X1	0.0566	1	0.0566	0.6864	0.4682	
	X2	9.23	1	9.23	111.90	0.0018	
	X3	0.2628	1	0.2628	3.19	0.1723	
Response Y3 (theoretical plates)	Quadratic model	1.045E+07	9	1.66E+06	69.99	0.0025	Significant
	X1	1458.00	1	1458.00	0.0879	0.7862	
	X2	1.657E+06	1	1.657E+06	99.86	0.0021	
	X3	58653.13	1	58653.13	3.53	0.1567	

Theoretical plates (Y_3) $=+5643.00-13.50A+455.12B+85.63C+56.75AB-158.25AC+871.50BC- 830.25A^2-1231.00B^2+160.00C^2$

The above quadratic model equations represent the effect of factors such as pH of the mobile phase, flow rate and acetonitrile concentration on responses tailing factor, retention time and theoretical plates. The "+" sign indicates a result that shows directly proportional whereas a "-" sign shows inverse relation. The representative plots for responses are presented in Figure 3 a-c, in which the interactivity between variables factors B and C and their mutual dependence is clearly observed, while factor A is kept constant. Figure 4 a-c represents that acetonitrile concentration in the mobile phase showed an extrusive effect on retention time. The retention time is increased with a decrease in acetonitrile concentration in the mobile phase and increased or decreased in pH of aqueous mobile phase; there is show no result on retention time. The increased flow rate with decreased retention time. Figure 5 confirmed that the theoretical plates of the column increased with increased flow rate and acetonitrile concentration in the mobile phase while the pH of the aqueous phase showed an insignificant effect on theoretical plates. The tailing factor decreased with an increase in acetonitrile concentration in the mobile phase and increased flow rate, while the pH of the aqueous phase did not show any significant effect on the tailing factor.

During numerical optimization shown in Figure 5 a-c parameters for the desirability were considered as pH,

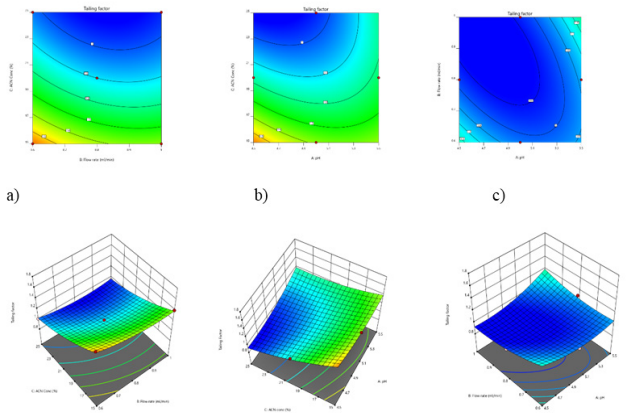

Figure 3 (a-c): Counter plots and 3-D response surface plots showing the influence of : a)pH, b)flow rate, c)% ACN concentration on tailing factor.

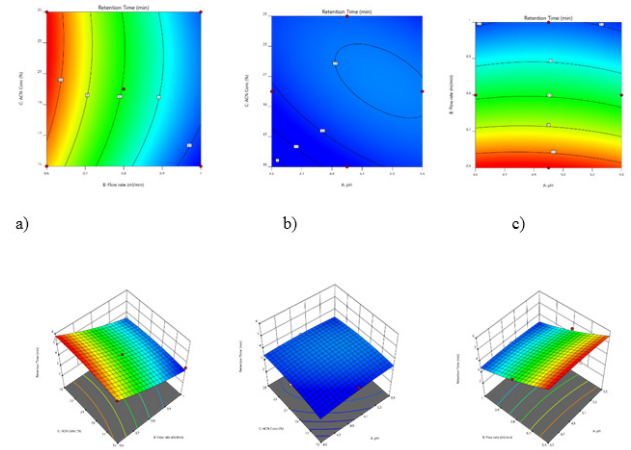

Figure 4 (a-c): counter plots and 3-D response surface plots showing the influence of : a)pH, b)flow rate, c)% ACN concentration on retention time.

flow rate, acetonitrile concentration, tailing factor, retention time, theoretical plates and combined. Here the desirability of all parameters found to be 100%. Hence the optimized chromatographic conditions, acetonitrile concentration (25%), the flow rate for column (1ml/min) and pH of aqueous mobile phase (5). As a result the retention time was found to be 3.516min, tailing factor 0.94 and the number of theoretical plates were 5902, the chromatogram of Azilsartan was shown in Figure 7.

Validation of method

The chromatographic separation was validated for linearity, range, accuracy, precision, robustness, specificity, system suitability according to (Q2 R1) ICH guidelines.[31]

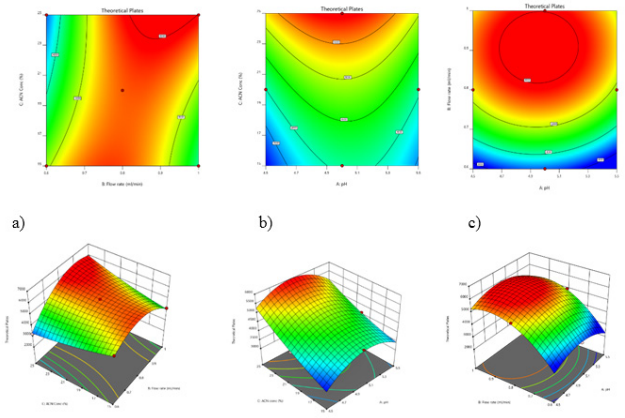

Figure 5 (a-c): Counter plots and 3-D response surface plots showing the influence of : a)pH, b)flow rate, c)% ACN concentration on number of theoretical plates.

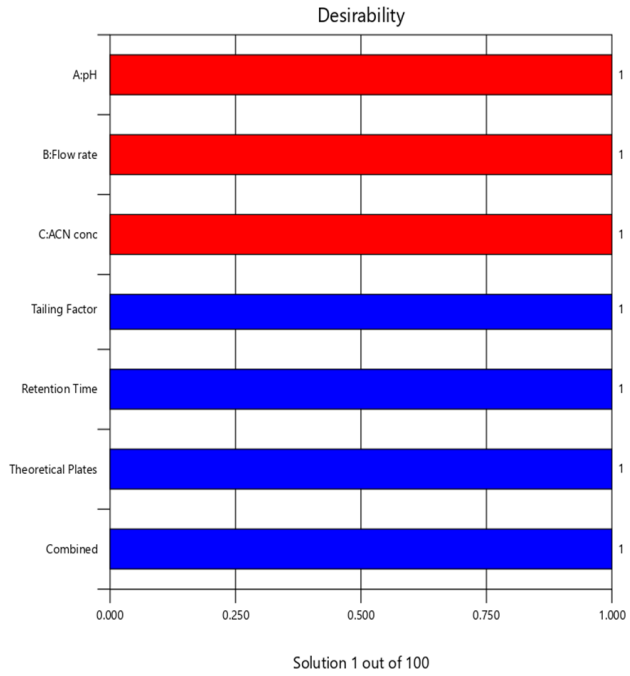

Figure 6: Desirability chart.

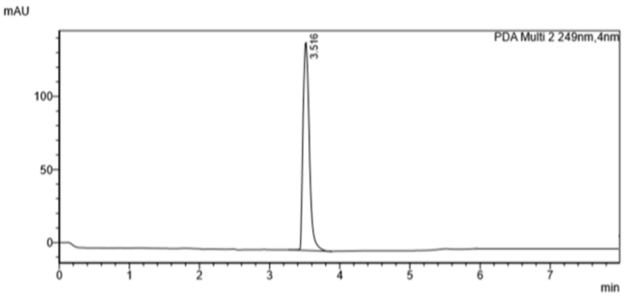

Figure 7: Chromatogram of Azilsartan (100µg/ml).

Table 4: Developed and optimized experimental condition				
Parameters	**Specification**	**Tailing Factor**	**Retention Time (min)**	**Theoretical Plates**
pH	5	0.94	3.516	5902
Flow rate	1ml/min			
ACN concentration	25%			

System suitability

For the system suitability study, the theoretical plates, peak area, tailing factor were studied. The acceptance criteria should meet the acceptance criteria. The six replicates of 20µg/ml of Azilsartan were injected results were shown in Table 5.

Table 5: System suitability	
Parameter	**Estimates**
USP Tailing	0.94
Plate count	5902
%RSD of Peak areas	0.59
Retention Time	3.516

Linearity and range

The five different serial concentrations were prepared for the standard calibration curve using acetonitrile in the rage of 5, 10, 15, 20, 25 and 30µg/ml. The linearity was performed by three replicate injections of each concentration. The regression equation was y=8898.3x+755.33 and the regression coefficient R^2=0.9997. It is shown in Figure 8.

Table 6: Intraday Precision				
Sr.No.	**Concentration(µg/ml)**	**Mean Peak Area**	**SD**	**%RSD**
1	15	133510	326.45	0.24
2	20	178059	795.66	0.44
3	25	225464	132.06	0.05

Precision

A repeatability study was studied within 6 replicate sets. The intraday study was performed by 3 replicates of 3 different i.e. 15, 20 and 25µg/ml of AZL was performed for intraday precision and interday precision (continuous 3 days) results were obtained. It was shown in Table 6, 7.

Table 7: Interday Precision				
Sr.No.	**Day**	**Concentration (µg/ml)**	**SD**	**%RSD**
1	Day1	15	142.68	0.10
	Day2			
	Day3			
2	Day1	20	171.34	0.09
	Day2			
	Day3			
3	Day1	25	665.37	0.29
	Day2			
	Day3			

Accuracy

Recovery study was performed by adding a known amount of drug corresponding to three concentrations, i.e. 50, 100 and 150% to the tablet formulation. It showed a satisfactory recovery of Azilsartan. The value of average % recovery and %RSD at each level was found within acceptance criteria that indicate the method is accurate. It was shown in Table 8.

Robustness

It is a measure of its robustness of an analytical procedure is a measure of its capability to remain unchanged by small, but intentional changes in method parameters and provides a signal that it was reliable in regular practice. There should be consistency of analysis with regarding for to planned variations in method parameters such as pH (4.5-5.5) and flow rate (0.8-1.2ml/min) shown in Table 9. The effect of each

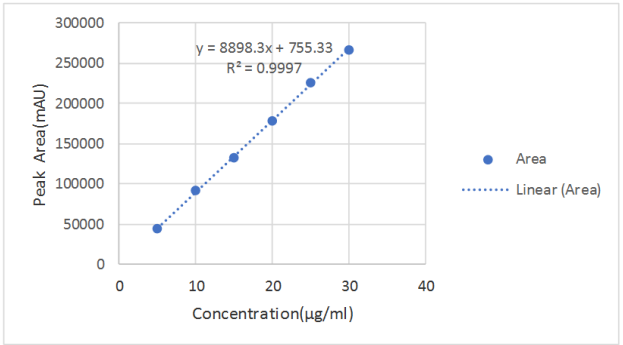

Figure 8: Calibration curve of Azilsartan.

Table 8: Accuracy and percent recovery validation of quantitative HPLC method of Azilsartan					
Levels(%)	**Amount of sample added(µg/ml)**	**Amount of standard added (µg/ml)**	**Amount recovered(µg/ml)**	**%Recovery**	**%RSD**
50	20	5	5.12	100.25	0.73
100	20	10	10.16	98.94	0.71
150	20	15	14.86	100.46	0.49

Table 9: Robustness study			
Parameter		**Retention Time (Rt)**	**%RSD of Peak Area**
Flow Rate (ml/min)	0.8	3.543	177685
	1	3.516	177795
	1.2	3.498	177432
	Mean	3.519	177637
	SD	0.022	186.13
	%RSD	0.62	0.104
pH	4.5	3.509	178452
	5	3.516	177543
	5.5	3.535	177623
	Mean	3.52	177872.7
	SD	0.013	503.3
	%RSD	0.28	0.29

Table 10: Summary of optimized RP-HPLC-PDA method.	
Parameter	**Results**
Regression coefficient	0.9996
Range	5-30µg/ml
Regression equation	y=8959.1x+46.5
Repeatability	0.59%(RSD)
Inter-day precision	0.09-0.29% (RSD)
Intra-day precision	0.05-0.44%(RSD)
Robustness	Robust
Specificity	Specific
Assay	99.66%

of the factor was anticipated and none of the factors go beyond the extreme and in this way proved the studied independent variables did not impact the results.

LOD and LOQ

The smallest amount can be detected and quantified by using the developed HPLC method. The detection limit was connected as a signal to noise ratio of 3:1 while quantification limit was specified as a signal to noise ratio 10:1. The %RSD was calculated.

Assay of marketed tablets of Azilsartan

The developed method was used for the estimation of azilsartan in oral tablet dosage form. The % assay was found to be 99.66% of Azilsartan from the tablet dosage form. It defines there was no interference and a high % recovery was found in the formulation excipients in the retention time of the drug shows the selectivity of the method for estimation of Azilsartan in the tablet dosage form.

In the preliminary experimental run Azilsartan was separated with trials using a mobile phase consisting of water and acetonitrile at pH 5 adjusted by orthophosphoric acid in ratio 80:20% v/v and flow rate 0.8ml/min. The drug was found to be retained, but the plate count was less and the tailing factor was more. Using the design of experiment approach the method was optimized using the mobile phase as water and acetonitrile in ratio 75:25% v/v, pH adjusted to 5 and flow rate 1ml/min. As a result, retention time was decreased with the improvement of theoretical plates and less tailing factor. By using the QbD approach the optimized mobile phase condition found that it was economical as well as got the responses with high accuracy and precision. The linearity was found within 5-30µg/ml and in precision, %RSD was found to be 0.59 which is less than 2. In this RP-HPLC method, the method was successfully validated in the optimized conditions and the validation parameters were within the limits. The results were shown in Table 10.

CONCLUSION

A reliable, precise, accurate, robust and cost-effective RP-HPLC method by the DoE approach using Box-Behnken design has been developed for the estimation of Azilsartan in bulk and pharmaceutical oral tablet dosage form. The systematic approach was utilized for method development which includes beginning with the determination of target profile characteristics, instrument qualification, risk assessment, design of experiment and validation. The design of experiment approach was successfully and effectively applied in the development of Azilsartan. The precision was found to be under acceptance criteria. There was no interference of excipients used in the formulation. According to ICH, USP and FDA guidelines, the method was validated. Hence, the outcome of this study will be very useful to researchers and industrial personnel.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest.

ABBREVIATIONS

HPLC: High Performance Liquid chromatography; **ICH:** International Conference on Harmonization; **CQA:** Critical Quality Attributes; **QbD:** Quality by Design; **ATP:** Analytical Target Profile; **SD:** Standard Deviation; **ANOVA:** Analysis of Variance; **PDA:** Photo diode array.

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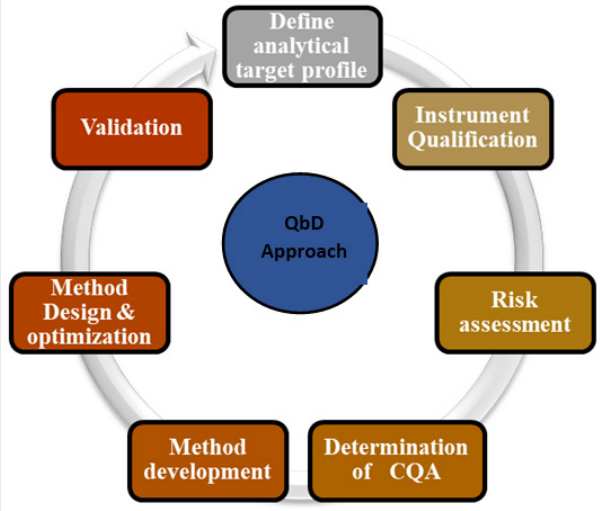
PICTORIAL ABSTRACT



SUMMARY

A reliable, sensitive, accurate and robust method was developed and optimized using the QbD approach with a high degree of effectiveness of requirements for estimation of Azilsartan in the tablet dosage form. Implementation of the QbD approach for chromatographic method development and optimization involves multiple steps. It consists of the first step of the determination of the target profile which sets the acceptance criteria. The second step consists of a review of possible risks (using the Ishikawa diagram) to identify the critical method parameters. The fourth phase includes based on risk assessment critical quality attributes were determined. The fifth phase consists of developing method. The sixth phase includes method optimizing using suitable design of experiments (DoE). The validation was performed according to ICH guidelines.

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