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Original scientific paper

Analytical Quality by Design approach in development and optimization of HPLC method for assay of angiotensin – converting enzyme inhibitor in tablets

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Abstract

Implementation of analytical quality by design approach is a helpful tool in development of a robust analytical method which is applicable throughout the lifecycle of the product. This model is perfect strategy for analytical method development, in order to reduce out-of-trend (OOT) and out-of-specification (OOS) results and to achieve high degree of robustness, providing cost-effective analytical method. The aim of this study was to implement a software which uses experimental design plans as an efficient and fast tool for development of method for quantitative determination of content of ACE inhibitor in tablets. Using statistical models in method development and optimization, the effect of each factor on responses of interest were calculated and the data were used to find the optimal chromatographic conditions. Best chromatographic separation within run time of 13 minutes was achieved on column Zorbax SB C18, 150 x 4.6 mm, (5 µm), using a mixture of 15 mM phosphate buffer pH 1.8 and acetonitrile as a mobile phase, with gradient elution and column temperature maintained at 60 °C. The validated method is specific, linear, accurate, precise and robust for the determination of angiotensin - converting enzyme inhibitor in tablets.

Key words: analytical quality by design, analytical method development and optimization, method validation, angiotensin – converting enzyme (ACE) inhibitor

Introduction

Medicines containing angiotensin-converting enzyme (ACE) inhibitor are commonly used to treat heart failure and high blood pressure and are often prescribed to people following a heart attack. These active pharmaceutical ingredients can reduce the activity of angiotensin-converting enzyme, responsible for hormones that help control the blood pressure (Herman et al., 2023). Since ACE inhibitor formulations are widely used, development of robust analytical methods for identification and quantification of API is mandatory. The active pharmaceutical ingredient, ACE inhibitor is a strong acid, with pKa value around 3.7. It is poorly soluble in water, due to its lipophilic nature (Remko M., 2007).

Compendial methods (British Ph., 2023; US Ph., 2020) for determination of content of ACE inhibitor in tablets are based on reversed phased liquid chromatography, isocratic elution on C18 column, with flow rate of 1.0 mL/min,

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using combination of sodium perchlorate, triethylamine and acetonitrile as mobile phase. Taking into consideration the complicated preparation of the mobile phase and the troubleshooting during method transfer, development of in-house method was needed.

Method development is a time consuming process especially when an one factor at time (OAFT) approach is used, due to its incapability to detect the interaction between each factors on desired chromatographic responses. This strategy has high risk in method failure, leading to repeat development and increasing the cost of the method.

Implementation of analytical quality by design approach can help in development of a robust analytical method which is applicable throughout the lifecycle of the product. This model is perfect strategy for analytical method development, in order to reduce out-of-trend (OOT) and out-of-specification (OOS) results and to achieve high degree of robustness, providing cost-effective analytical method. By using statistical models in method development and optimization, the effect of each factor on responses of interest can be easily calculated and the data can be used to find the optimal chromatographic conditions.

The aim of this study was to implement a software which uses experimental design plans as an efficient and fast tool for development of method for quantitative determination of content of ACE inhibitor in tablets.

Material and methods

Material and Equipment

Generic product sample (tablets containing 5 mg ACE inhibitor), working standard of the active pharmaceutical ingredient, impurity A, B, C, D, E, F, M, K and O standards (CRS) were used. All the reagents used: potassium dihydrogen phosphate (Merck), acetonitrile (Merck), methanol (Merck), orto-phosphoric acid (Merck), were HPLC grade.

Experiments were performed on Agilent 1290 Infinity Automated Method Development system and Dionex UltiMate™ 3000 (Thermo Scientific) system. All data were processed and evaluated with Fusion and MODDE software.

Chromatographic conditions

Columns XBridge C18 150 x 4.6mm (5µm), Zorbax SB C18 150 x 4.6 mm (5 µm), Zorbax SB CN 250 x 4.6 mm (5 µm) and Zorbax 300 SB C18 250 x 4.6 mm (5 µm) were evaluated during screening and optimization phase, using phosphate buffer and acetonitrile or methanol in the mobile phases, with flow rate from 0.8 mL/min – 2.0 mL/min. Column temperature was maintained from 25 - 65 °C. Final method was performed on column Zorbax SB C18 150 x 4.6 mm, 5 µm particle size, column temperature set to 60°C, with gradient elution, using 15 mM phosphate buffer pH 1.8 as mobile phase A and acetonitrile as mobile phase B. Total analysis time was 13 minutes. The volume of injection of standard and sample solutions was 10 µL. Detection was performed on 210 nm.

Preparation of standard solutions

API standard solution: 25 mg API working standard was dissolved in 100.0 mL diluent. 5.0 mL of the stock solution were further diluted to 25.0 mL with the same diluent (concentration 0.05 mg/mL);

Impurity standard solution: 2.5 mg impurity CRS standard was dissolved in 25.0 mL diluent (concentration 0.1 mg/mL).

Preparation of sample solution: ten (10) tablets were dissolved and diluted to 1000.0 mL with the diluent and filtered through 0.2 µm regenerated cellulose membrane filter before injecting, discarding the first 2 mL of the filtrate.

Analytical method validation: Method was validated for specificity, linearity, accuracy, precision, stability of the solutions and filter study (ICH guideline Q2 (R1), 2005).

Specificity: diluent, placebo solution (prepared in the same manner as described for the API), standard and sample solutions were prepared and injected into HPLC as per methodology.

Table 1. Experimental variables – Fusion column and solvent screening phase

Type of column	XBridge C18 150x4.6mm (5µm) /Zorbax SB C18 150x4.6mm (5µm)/ Zorbax SB CN 250x4.6mm (5µm) /.Zorbax 300 SB C18 250x4.6mm (5µm)
pH of buffer	pH 1.5 (50 mM KH_2PO_4) / pH 2.5 (50 mM KH_2PO_4)
Type of organic solvent	acetonitrile / methanol
% of organic solvent	15% – 50%
Flow rate	0.8mL/min – 2.0mL/min
Column temperature	25 °C – 65 °C

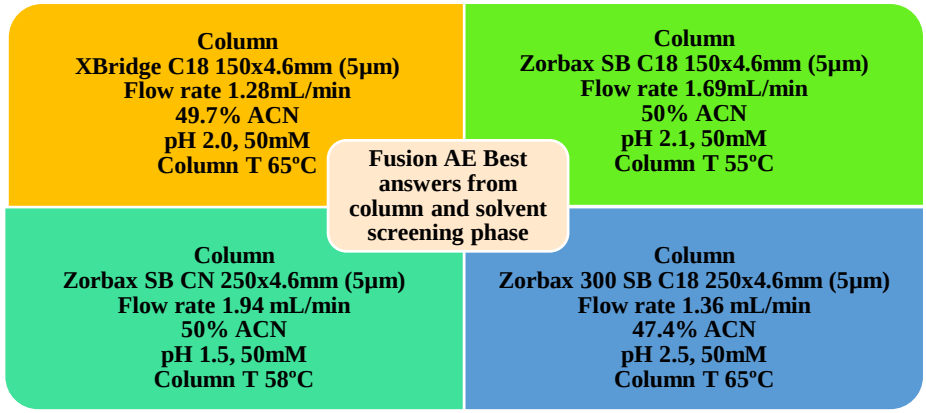

Fig. 1. Best answers with acetonitrile as organic solvent – Fusion column and solvent screening phase.

Accuracy: amount of placebo was taken separately into different flasks and spiked with known quantities of ACE inhibitor active ingredient at three different levels – 0.025 mg/mL; 0.050 mg/mL and 0.075 mg/mL, in triplicate.

Linearity: series of standard solutions were prepared at seven different concentrations that span from 0.010 mg/mL to 0.075 mg/mL API. Following standard concertations were evaluated: 0.010 mg/mL; 0.020 mg/mL; 0.025 mg/mL; 0.040 mg/mL; 0.050 mg/mL; 0.060 mg/mL and 0.075 mg/mL.

Precision: six sample solutions were prepared individually, using same batch of tablets. Samples were prepared by different analysts, on different equipment and on different day.

Stability: standard and sample solution were prepared and analyzed at initial point and at different time intervals by keeping the solutions at 4°C.

Filter recovery: loss of API from the standard solution when passing through 0.2 µm regenerated cellulose membrane filter, was determined by comparison of the peak areas from chromatograms obtained after injection of six individually filtered aliquots of standard solution in concentration of 0.05 mg/mL with the peak area from chromatograms obtained after injection of unfiltered standard solution.

Results and discussion

Fusion screening and optimization phase

Taking into account that API is soluble only in solutions with low pH, two pH ranges were evaluated (Table 1), and four different columns stable at the combination of low pH and high temperature. Phosphate buffer was chosen, based on its routine use in chromatography.

Testing was performed with Fusion experiments for column and solvent screening and optimization (Turpin et al., 2008). All experimental variables are given in Table 1.

Model design with 82 analyzis was generated.

All experimental data were properly integrated and next assumptions were made:

- When methanol was used as organic solvent, elution of main peak was late and high column pressure was observed;
- Column temperature showed significant influence on tailing factor and number of theoretical plates (NTP) of the main peak;
- The percent of acetonitrile in the mobile phase had major impact on the peak shape.

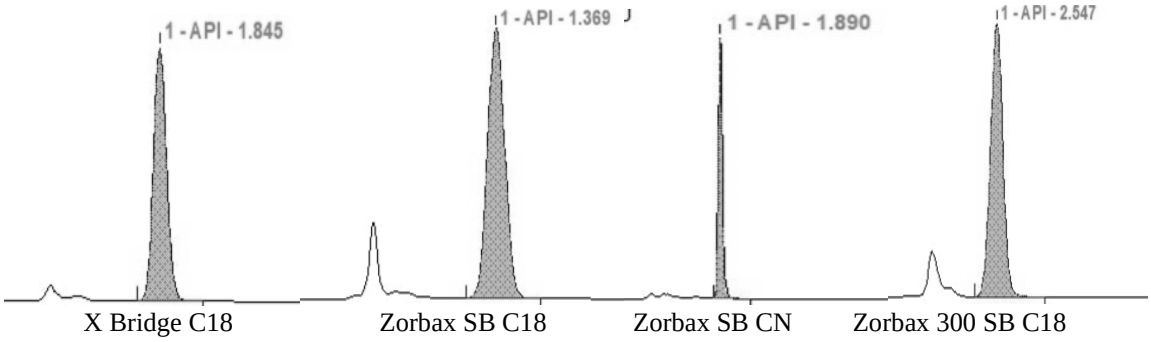

Fig. 2. Best answers – Fusion column and solvent screening phase.

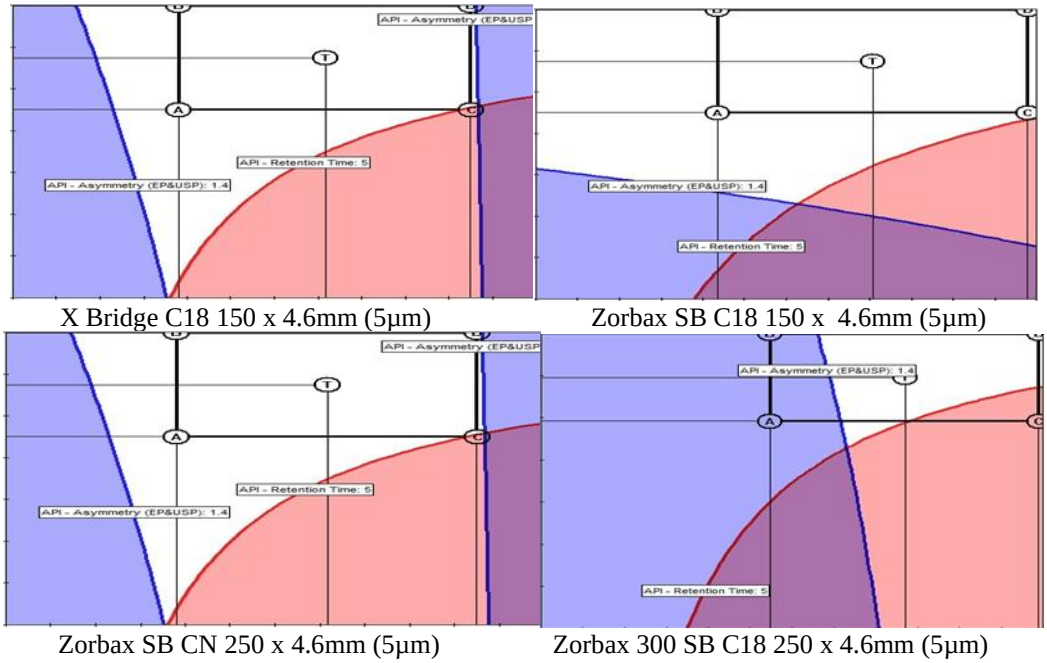

Fig. 3. Acceptable Performance Region – Fusion column and solvent screening phase.

In order to generate best statistical answer for this experimental stage, acceptance criteria for retention time and tailing were set. Number of theoretical plates were not evaluated in this phase. For tailing factor, the value of 1.0 was set as a target value with the limits from 0.96 to 1.4. For retention time, option *minimize* (faster elution of the main peak) was selected with the limit of 5 minutes.

All chromatographic parameters were calculated using a software statistical tool and eight best answers were generated for each column and for both types of organic solvent, as best combination of experimental variables. Taking into account the better elution power of acetonitrile, methanol was not considered further in the study. Best answers with acetonitrile are represented on Fig. 1.

Best answers were analyzed and Fusion statistical prediction for good chromatography were confirmed (Fig. 2).

For visualization, the influence of varied method parameters on best answer's chromatography *Acceptable Performance Region* is presented on Fig. 3. Region with white color represents the *Final Design Space* and cover the part where settled acceptance criteria for retention time and tailing are fulfilled. Regions with blue or red color, represent combination of method parameters that do not fulfill settled acceptance criteria (Fukuda et al., 2018; Little, 2013; Turpin et al., 2008).

Experimental variables on all four columns are temperature (x) and percent of acetonitrile (y), while flow rate and pH value are constant variables, according to the generated best answers. *Proven Acceptable Range (PARs)* rectangle are added on the plots on Fig. 3 (Little, 2013). Corners and target point are the recommended method conditions, as high value for percent of acetonitrile and column temperature.

Acceptance Performance Region for Zorbax 300 SB C18 250 x 4.6 mm (5 µm) column indicate that this column is less robust than other columns and was omitted from further evaluation. Column Zorbax SB CN 250 x 4.6 mm (5 µm) was also excluded due to its specific mechanism of separation.

Fusion optimization, as a next step following column and solvent screening, was accomplished with additional 67 analyzis, on columns X Bridge C18 150 x 4.6 mm (5µm) and Zorbax SB C18 150 x 4.6 mm (5 µm) and acetonitrile as a type of organic solvent. All experimental variables are given in Table 2.

Table 2. Experimental variables – Fusion optimization phase

Type of column	XBridge C18 150x4.6mm (5µm) / Zorbax SB C18 150x4.6mm (5µm)
pH of buffer	pH 1.5 (50 mM KH_2PO_4) / pH 2.0 (50 mM KH_2PO_4)
% of acetonitrile	30% – 50%
Flow rate	1.1mL/min – 1.7mL/min
Column temperature	30 °C – 50 °C

Table 3. Settled acceptance criteria – Fusion optimization phase

Retention time	Target – 5 min.	Lower bound – 3 min.	Upper bound – 5.5 min.
Tailing factor	Target – 1.0	Lower bound – 0.99	Upper bound – 1.25

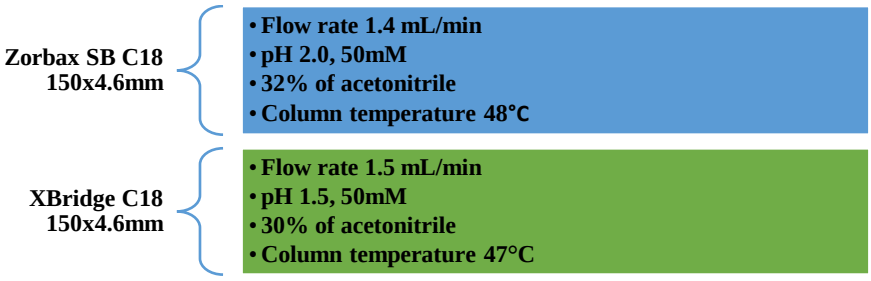

Fig. 4. Best answers – Fusion optimization phase.

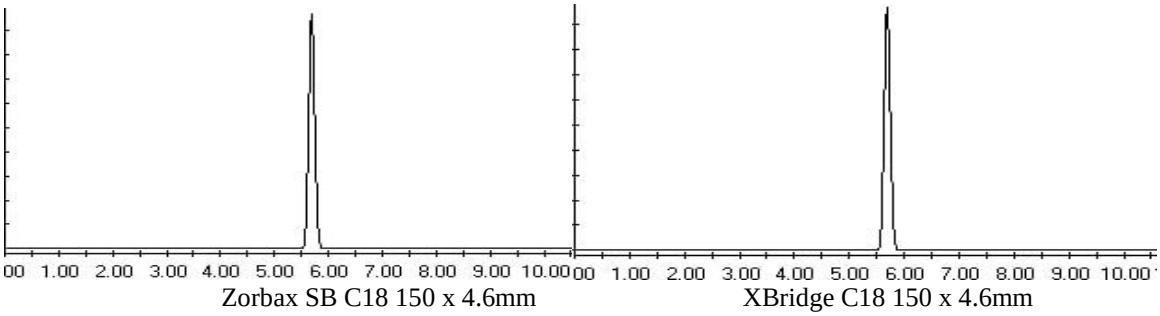

Figure 5. Predicted chromatography of best answers – Fusion optimization phase.

Table 4. Predicted response values – Fusion optimization phase

	Zorbax SB C18 150x4.6mm	**XBridge C18 150x4.6mm**
Predicted retention time	4.86	5.69
Predicted tailing factor	1.10	1.10

Responses of interest in Fusion optimization stage, were tailing factor of the peak of API and retention time (Table 3), with stricter limits than screening phase.

Two similar best answers were generated, one for each column, given in Fig. 4.

Using Point Prediction tool, Fusion predicted the chromatography and responses of interest (Fig. 5 and Table 4).

Both analyzed answers, given in Figure 6, were satisfactory and comparable with the predicted chromatography. Nevertheless, by using Zorbax SB C18 150 x 4.6 mm column better peak shape and higher NTP (around 2100) were obtained, compared to XBridge C18 column. Additional information about the design space of method on column Zorbax SB C18 150 x 4.6 mm were obtained by evaluation of *Acceptable Performance Region*, represented on Fig. 7

The white region on Fig. 7 is the *Final Design Space*, where retention time of peak and tailing factor were within the defined limits (Fukuda et al., 2018). These values were not obtained using lower column temperature. The percent of acetonitrile had major impact on peak responses and the best chromatographic separation was obtained with combination of lower flow rate and higher percentage of acetonitrile (up to 39%), or higher flow rate and lower percentage of acetonitrile (32%). pH value of buffer had no influence on the chromatographic separation.

The dependence of the method parameters and peak responses was also visualized through *Multiple Response Effect Plot* (Fig. 8). Changing the flow rate and the percent of acetonitrile, did not influenced the tailing factor value, but retention time was increasing when lower percentage of acetonitrile was used (Fukuda et al., 2018).

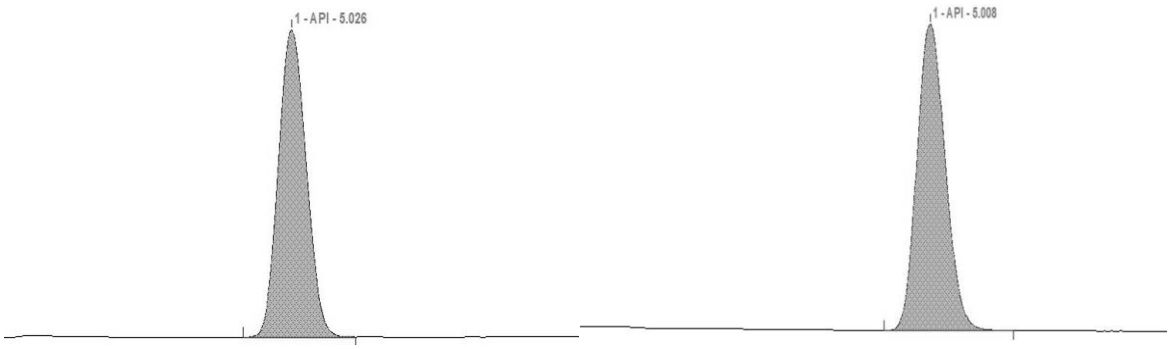

Fig. 6. Analyzed chromtography of best answers – Fusion optimization phase.

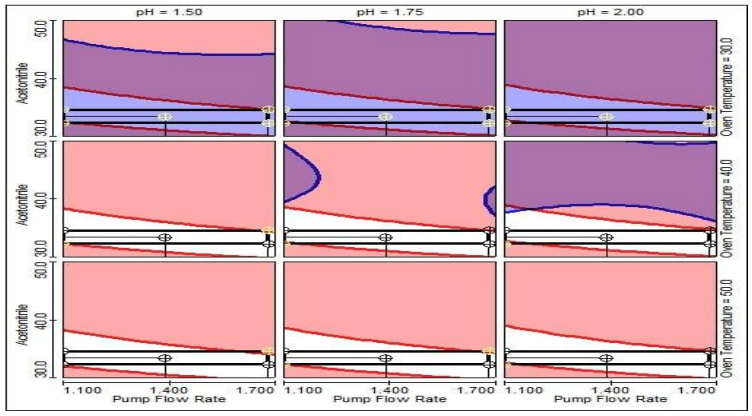

Fig. 7. Acceptable Performance Region on column Zorbax SB C18 – Fusion optimization phase.

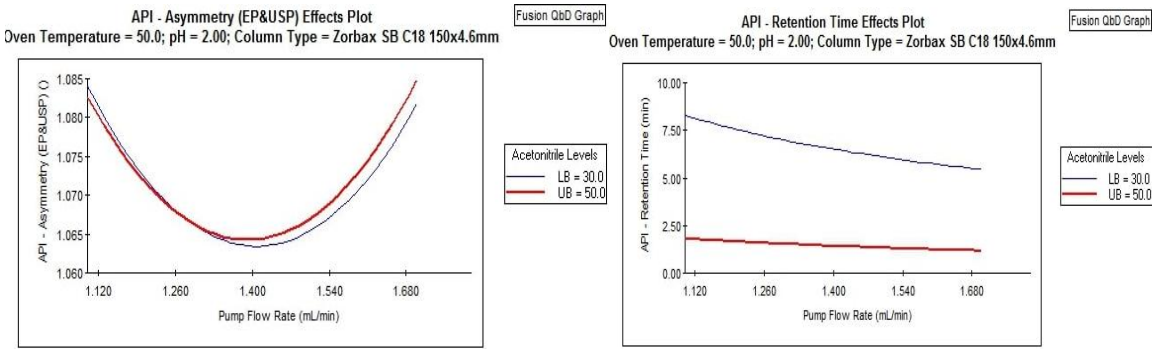

Fig. 8. Multiple Response Effects plot for influence of flow and acetonitrile – Fusion optimization phase.

Combination of high molarity (50 mM) of buffer, low pH value (2.0) and high temperature (50 ºC) represents risk for fast ruining column silica. Furthermore, the best answer provided by Fusion was analyzed with 30 mM buffer. It was noticed that the retention time and tailing factor of peak were unaffected, however the number of theoretical plates decreased bellow 2000.

MODDE Optimization phase – isocratic method

Following step was to improve the NTP of peak of API using software MODDE, full factorial design (Fukuda et al., 2018; Kumar and Sangeetha, 2020; Puranpole et al., 2016). Optimization was initiated using column Zorbax SB C18, with combination of 30 mM buffer and acetonitrile as a mobile phase (Table 5).

MODDE proposed matrix design with 16 experiments, which were completed on Agilent 1290 Infinity Automated Method Development system (Table 6). Parameters of interest in this optimization were tailing factor, retention time and NTP of the peak of API.

Influence of method variables and their possible interactions were calculated and presented through graphics and diagrams (Fukuda et al., 2018; Ganorkar and Shirkhedkar, 2017; Puranpole et al., 2016).

Table 5. Experimental variables – MODDE optimization of isocratic method

pH of buffer	pH 1.8 (30 mM KH_2PO_4) / pH 2.2 (30 mM KH_2PO_4)
% of acetonitrile	30% / 34%
Flow rate	1.1mL/min / 1.7mL/min
Column temperature	40 °C / 50 °C

Table 6. Chromatographic conditions and responses – MODDE optimization of isocratic method

Experiment name	Column Temp.	pH	Flow rate	%ACN	Tailing factor	Retention time	NTP
N1	40	1.8	1.1	30	1.16	8.10	1700
N5	40	1.8	1.7	30	1.18	5.46	1282
N17	40	1.8	1.1	30	1.17	8.10	1701
N18	40	1.8	1.1	30	1.16	8.10	1664
N19	40	1.8	1.1	30	1.16	8.10	1684
N2	50	1.8	1.1	30	1.14	7.81	3018
N6	50	1.8	1.7	30	1.15	5.18	2545
N9	40	1.8	1.1	34	1.21	4.70	1970
N10	40	1.8	1.7	34	1.21	3.17	1360
N13	50	1.8	1.1	34	1.15	4.66	3024
N14	50	1.8	1.7	34	1.11	3.10	2465
N3	40	2.2	1.1	30	1.12	8.42	1128
N7	40	2.2	1.7	30	1.12	5.70	809
N4	50	2.2	1.1	30	1.09	8.27	1808
N8	50	2.2	1.7	30	1.09	5.52	1489
N11	40	2.2	1.1	34	1.12	4.95	1046
N15	40	2.2	1.7	34	1.14	3.33	711
N12	50	2.2	1.1	34	1.09	4.95	1560
N16	50	2.2	1.7	34	1.14	3.33	711

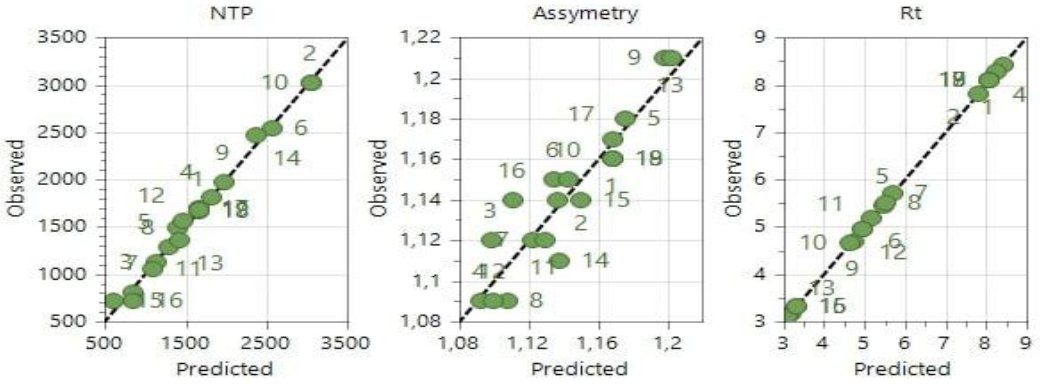

Fig. 9. Observed vs Precited Reponses Plot – MODDE optimization of isocratic method.

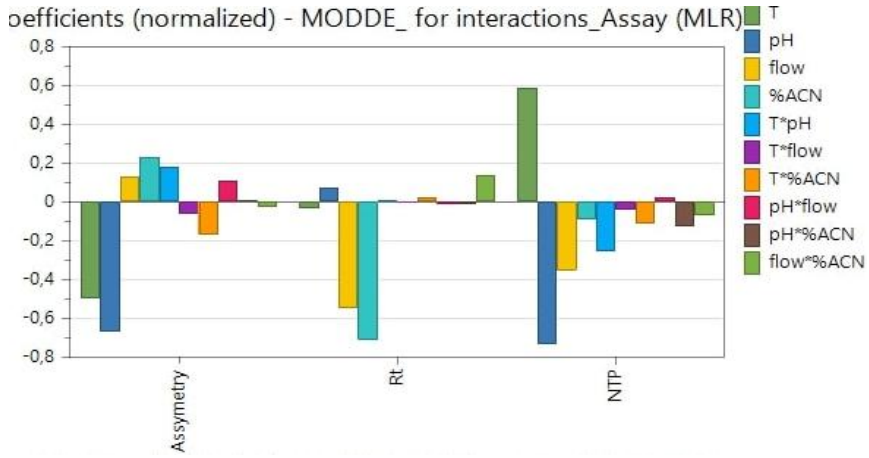

Fig. 10. Pareto diagram for method parameter influence and interaction – MODDE optimization of isocratic method.

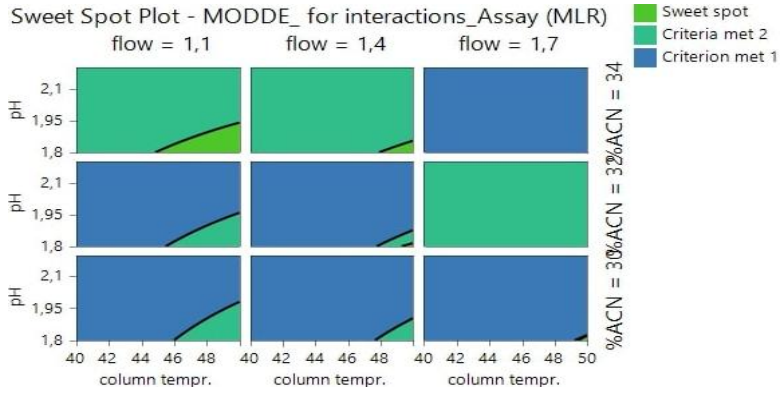

Fig. 11. Sweet Spot Plot – MODDE optimization of isocratic method.

The plot on Fig. 9 displays the observed versus predicted values of the response (Ganorkar and Shirkhedkar, 2017). With a good model, all points would fall on the 1:1 line. Model for NTP and retention time are examples for a good model, but for tailing factor some outliers were detected. Taking into consideration that all experimental values of tailing factor are in acceptable ranges, the outliers were insignificant and the model was considered acceptable.

Pareto Diagram on Fig. 10 displays the coefficients for all responses (Fukuda et al., 2018; Ganorkar and Shirkhedkar, 2017). For a parameter to have significant influence on chromatography, the value on the scale should be around 1. Therefore, the variable interaction had no influence on chromatographic parameters, only the individual method condition. From the *Pareto Diagram* it could be concluded that low values for pH and temperature were needed for improving the peak tailing factor. For faster peak elution, as previously concluded, higher values of flow rate and percent of acetonitrile are required.

Sweet Spot Plot in Fig. 11, provide information about which combination of method conditions is needed for NTP value higher than 2500, retention time between 3.5 and 5.2 minutes and the value of tailing factor between 1.0 and 1.2. All values in the light green area were satisfactory (pH 1.8, temperature 45 – 50 ºC, flowrate 1.1 mL/min and of 34% of acetonitrile).

For statistical calculation on best parameter combination, tool *Optimizer* was used. Target values for the retention time was 4 to 5 minutes and for NTP was 3000 to 4000. Tailing factor was not considered in this phase, due to its satisfactory results in all experiments.

MODDE's best answer and predictions for peak responses of interest are given in Table 7.

Best answer was analyzed (Fig. 12) and estimates were confirmed, peak eluted on 4.5 minutes with NTP value of 4068.

Since optimized isocratic method was achieved, solution with mixture of known impurities (impurity A, B, C, D, E, F, M, K and O) was analyzed to confirm specificity (Chaudhari and Shirkhedkar, 2019; ICH guideline Q2 (R1), 2005). No interference was observed with the main peak. All detected peaks of process impurities were eluted in same run time as the main peak, except impurity O which was not detected. Degradation impurity D was eluted 3 runs later, however with no interferences of peak of API.

Robustness of isocratic method was checked. Different HPLC systems and different column batches

were evaluated, with small deliberate changes of the final parameters. (Chaudhari and Shirkhedkar, 2019; ICH guideline Q2 (R1), 2005). Satisfactory results were obtained. Subsequently, isocratic method would be suitable for routine use in quantification of API in samples, but during stability study, a problem with chromatography was observed on a different configuration of binary HPLC system. It was noticed that in samples kept at accelerated conditions of 40 ºC, 75% RH, for 3 months, where degradation impurity D is increased to 6%, bad peak shape of API in the 4th run was observed, indicating that the peak of impurity D after 4th run co-eluted with the peak of API. Therefore, new method optimization was necessary.

MODDE Optimization phase – gradient method

The purpose of this phase was elution of impurity D in identical run time as peak of ACE inhibitor.

Gradient elution described in Table 8 was evaluated as starting point for improvement. Method parameters until 6 minutes were unaffected.

MODDE full factorial model for optimization, generated matrix design with 29 experiments (Fukuda et al., 2018; Kumar and Sangeetha, 2020; Puranpole et al., 2016). In this design, a standard solution with mixture of all impurities, was evaluated to ensure no interferences in the new gradient method with the main peak. Experiments were carried out on Dionex UltiMate™ 3000 (Thermo Scientific) system. Experimental variables are given in Table 9.

During data evaluation it was noticed that resolution between the main peak and two process impurities A and M was significantly affected. In all experiments, satisfactory resolution between the main peak and degradation product, impurity D, was observed. To obtain ideal chromatography, responses of interest in this phase were: retention time and NTP of ACE inhibitor, retention time of degradation impurity D and resolution between main peak and process impurities A and M (Table 10).

Table 7. Best answer prediction – MODDE optimization of isocratic method

Column T	pH	Flow rate	%ACN	RT	NTP
50	1.8	1.22	34	4.3	2920

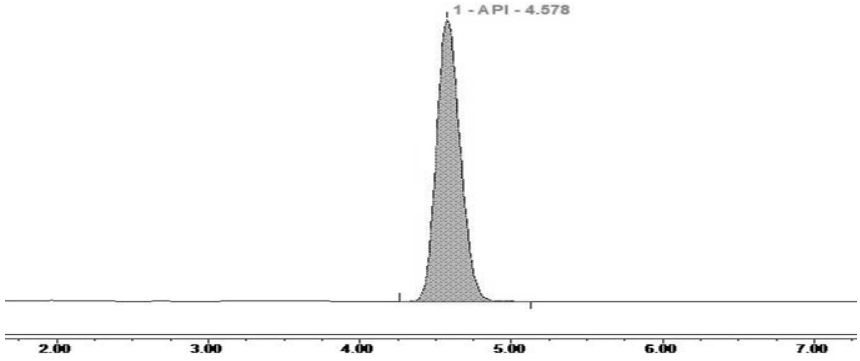

Fig. 12. Analyzed chromatogram according best answer – MODDE optimization of isocratic method.

Table 8. Gradient elution for optimization – MODDE optimization of gradient method

Minutes	pH 1.8	Acetonitrile	Flow rate
0	66	34	1.1 mL/min
6	66	34	1.1 mL/min
7	45	50	2.0 mL/min
11	45	50	2.0 mL/min
11.5	66	34	1.1 mL/min
13	66	34	1.1 mL/min

Table 9. Experimental variables – MODDE optimization of gradient method

pH of buffer	pH 1.6 / pH 2.2
Buffer molarity	10 mM / 30 mM
% acetonitrile for elution of impurity D	45% / 55%
Flow rate for ACE inhibitor elution	0.9 mL/min / 1.3 mL/min
Column temperature	50 °C / 60 °C

Table 10. Chromatographic responses – MODDE optimization of gradient method

Experiment name	Retention time API	NTP API	Retention time Imp.D	Resolution API/Imp.M	Resolution API/Imp.A
N18	4.98	7435	9.27	6.98	4.98
N21	3.49	4492	10.86	5.60	3.49
N16	5.15	3769	11.00	5.40	5.15
N17	5.23	2548	9.28	4.19	5.23
N25	3.70	2820	9.00	4.15	3.70
N15	4.96	5997	11.24	6.44	4.96
N19	4.81	7459	9.16	6.90	4.81
N23	3.40	6029	10,54	6.24	3.40
N24	3.53	4581	9.10	5.65	3.53
N20	5.45	3476	9.18	4.74	5.45
N22	3.89	1783	10.90	3.95	3.89
N26	3.90	2694	9.00	4.16	3.90
N27	3.90	2757	9.00	4.24	3.90
N28	3.89	2742	9.00	4.24	3.89
N29	3.89	2738	9.00	4.23	3.89
N2	5.08	7783	11.60	7.08	5.08
N4	5.00	5969	9.39	6.37	5.00
N11	3.57	4437	9.22	5.71	3.57
N13	3.50	4959	9.13	6.58	3.50
N1	5.27	2427	11.89	4.01	5.27
N6	5.24	3517	9.30	4.83	5.24
N9	3.72	2790	10.86	4.26	3.72
N12	3.75	1819	9.25	3.37	3.75
N7	4.97	7823	9.29	7.08	4.97
N8	3.64	4684	11.15	5.74	3.64
N10	3.53	6143	10.85	6.26	3.53
N3	5.64	3595	11.34	4.86	5.64
N5	5.64	2407	9.42	4.70	5.64
N14	4.03	2708	9.14	4.22	4.03

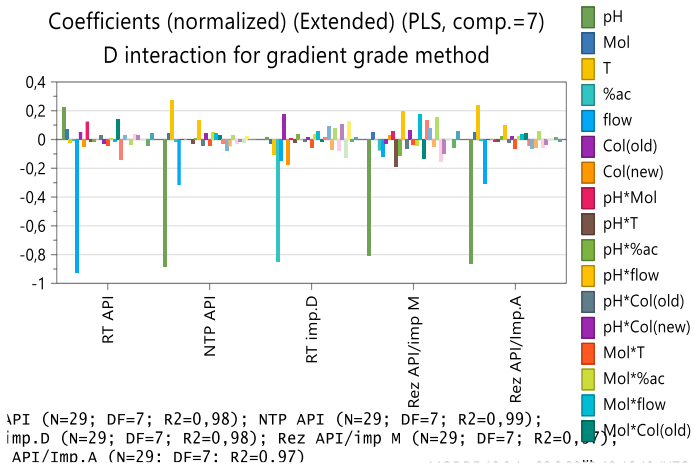

Fig. 13. Pareto Diagram for method parameter influence and interaction – MODDE optimization of gradient method.

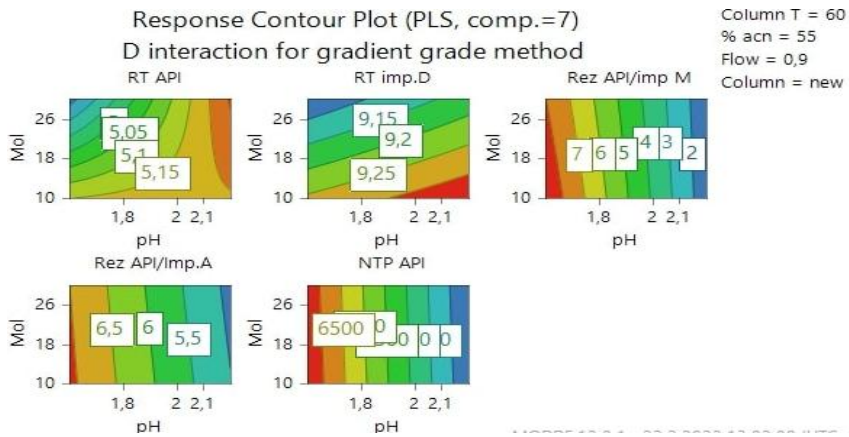

Fig. 14. Response Contour Plot – MODDE optimization of gradient method.

From *Pareto Diagram* on Fig. 13, it can be concluded that low pH values are needed to obtain better resolution between ACE inhibitor peak and process impurities A and M. The retention time of impurity D was affected only by the portion of acetonitrile.

Response Contour Plot in Figure 14 confirms that satisfactory response values were obtained on low pH, high column temperature, high percentage of acetonitrile and low flow rate.

For best answer calculation, in tool *Optimizer* following limits were defined: 4.5 minutes as a target for a retention time of ACE inhibitor; 9 minutes as a target for retention time of impurity D, maximum NTP from 4000-6000 and 4.0 as a target resolution between the main peak and impurity A and M.

The best results were obtained when column temperature was maintained at 50ºC (Table 11). From design experiments it is known that this parameter has positive influence on peak shape and NTP, so the final method would be same as best answer, only with column temperature of 60 ºC. The final gradient elution proposed for the method is:

0–6 minutes - 66% pH 1.8 : 34% acetonitrile, flow rate 1.1 mL/min

6 -7 minutes - 45% pH 1.8 : 55% acetonitrile, flow rate 2.0 mL/min

7-11 minutes - 45% pH 1.8 : 55% acetonitrile, flow rate 2.0 mL/min

11-11.5 minutes - 66% pH 1.8 : 34% acetonitrile, flow rate 1.1 mL/min

11.5-13 minutes - 66% pH 1.8 : 34% acetonitrile, flow rate 1.1 mL/min.

Robustness of final method with MODDE Placket-Burman design

Robustness of gradient method was confirmed using Plackett-Burman design matrix, with 11 experiments performed on Agilent 1290 Infinity Automated Method Development system (Fukuda et al., 2018; Maskovic et al., 2010). Small and deliberate changes of method parameters are given in Table 12.

System suitability parameters of interest were retention time, tailing and NTP of peak of ACE inhibitor and resolution between main peak and impurity D (Table 13).

Table 11. Best answer – MODDE optimization of gradient method

Column T	pH	Molarity	Flow rate	%ACN
50	1.8	15	1.1	55

Table 12. Experimental variables – Placket–Burman robustness

pH and molarity of buffer	13.5mM pH 1.6 / 13.5mM pH 2.0 /16.5mM pH 1.6 / 16.5mM pH 2.0
% acetonitrile for elution of ACE inhibitor	32% / 36%
Flow rate for ACE inhibitor elution	0.9 mL/min / 1.3 mL/min
Column temperature	55 °C / 65 °C
Different column batch	New / Old column

Table 13. Design matrix – Placket–Burman robustness

Experiment name	Flow rate	% ACN	Column T	Molarity	pH	Column	Retention time API	NTP	Tailing factor	Resolution
N2	1.3	36	55	13.5	2.0	old	2.98	2289	1.18	31.50
N4	0.9	36	65	16.5	1.6	old	3.99	8119	1.31	33.27
N5	1.3	32	65	16.5	2.0	old	4.49	3289	1.16	20.58
N8	0.9	32	55	13.5	1.6	old	6.11	7520	1.19	14.76
N9	0.9	32	55	13.5	1.6	old	6.11	7416	1.19	14.71
N10	0.9	32	55	13.5	1.6	old	6.09	7149	1.20	14.60
N11	0.9	32	55	13.5	1.6	old	6.09	7141	1.20	14.60
N1	1.3	32	55	16.5	1.6	new	4.19	3696	1.26	23.91
N3	1.3	36	65	13.5	1.6	new	2.72	5064	1.28	44.08
N6	0.9	36	55	16.5	2.0	new	4.24	3094	1.26	21.64
N7	0.9	32	65	13.5	2.0	new	6.15	6068	1.17	10.84

Influence of the small method changes on desired chromatographic responses is visualized and discussed through graphics as Summary of Fit, Sweet Spot Plot and Response Surface Plot. Statistical model *Summary of Fit*, represented on Fig. 15, displays four columns for each response. R2 measures how well the model fits the data. A large R2 (> 0.8) is a necessary for a good model. Q2 tells how well the model predicts new data. A useful model should have a large Q2 (> 0.5). Reproducibility is the variation of the response under the same conditions, compared to the total variation of the response. Model validity is a measure of the validity of the model. When the model validity bar is larger than 0.25, there is no lack of fit of the model. In this case, a validity model for responses' retention time and resolution, is displayed with negative bars. However, this is an example for a good model (Q2 > 9) where validity might have low value due to high sensitivity in the test or extremely good replicates.

On Fig. 16, robustness of method is presented with *Sweet Spot Plot*, where green colored area demonstrates that method is robust on all combination of evaluated molarity, flow rate, column temperature and % of acetonitrile.

The *Response Surface Plot* on Fig. 17, demonstrates the effect of flow rate and % of acetonitrile on resolution and NTP. Molarity, pH and column temperature are constant values. Yellow area represents the resolution, whereas the green area represents NTPs. Resolution is decreasing and NTPs are increasing on higher value of flow rate and % of acetonitrile.

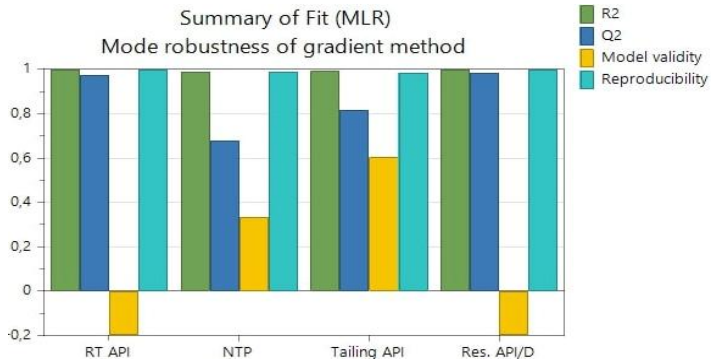

Fig. 15. Summary of Fit – Plackett–Burman robustness matrix design.

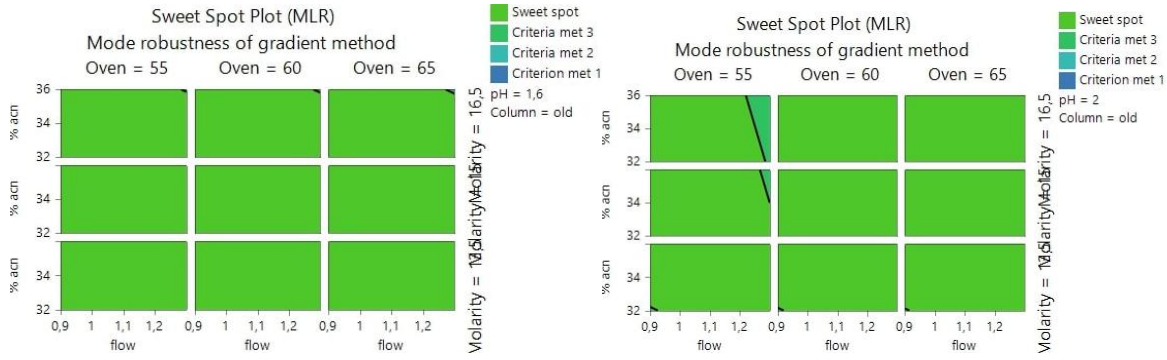

Fig. 16. Sweet Spot Plot – Plackett–Burman robustness matrix design.

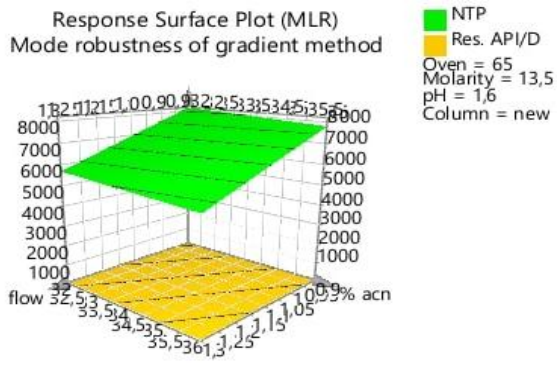

Fig. 17. Response Surface Plot – Plackett–Burman robustness matrix design.

Finally, it can be concluded that optimized gradient method is robust for all responses of interest. With change of pH, molarity, flow rate and % of acetonitrile, retention time of peaks and tailing are insignificantly affected.

Analytical method validation

Method was validated for Specificity, Linearity, Accuracy, Precision, Stability of the solutions and Filter study (ICH guideline Q2 (R1), 2005).

Typical chromatograms for specificity of the method are shown in Fig. 18, indicating that no interference of the diluent and placebo peaks with the peak of ACE inhibitor was found.

Recovery values obtained from determination of API in the range from 50 to 150 % (0.025 mg/mL – 0.075 mg/mL) are between 98.0 % and 102.0 % and RSD is not more than 2.0 %. Results in Table 14 indicate that the test method has an acceptable level of accuracy.

The results obtained from the regression analysis of the peak area versus concentration data (Table 15 and Fig. 19) indicate that method is linear (correlation coefficient > 0.999) in the concentration range from 20% to 150% of the standard's working range (0.010 - 0.075 mg/mL API).

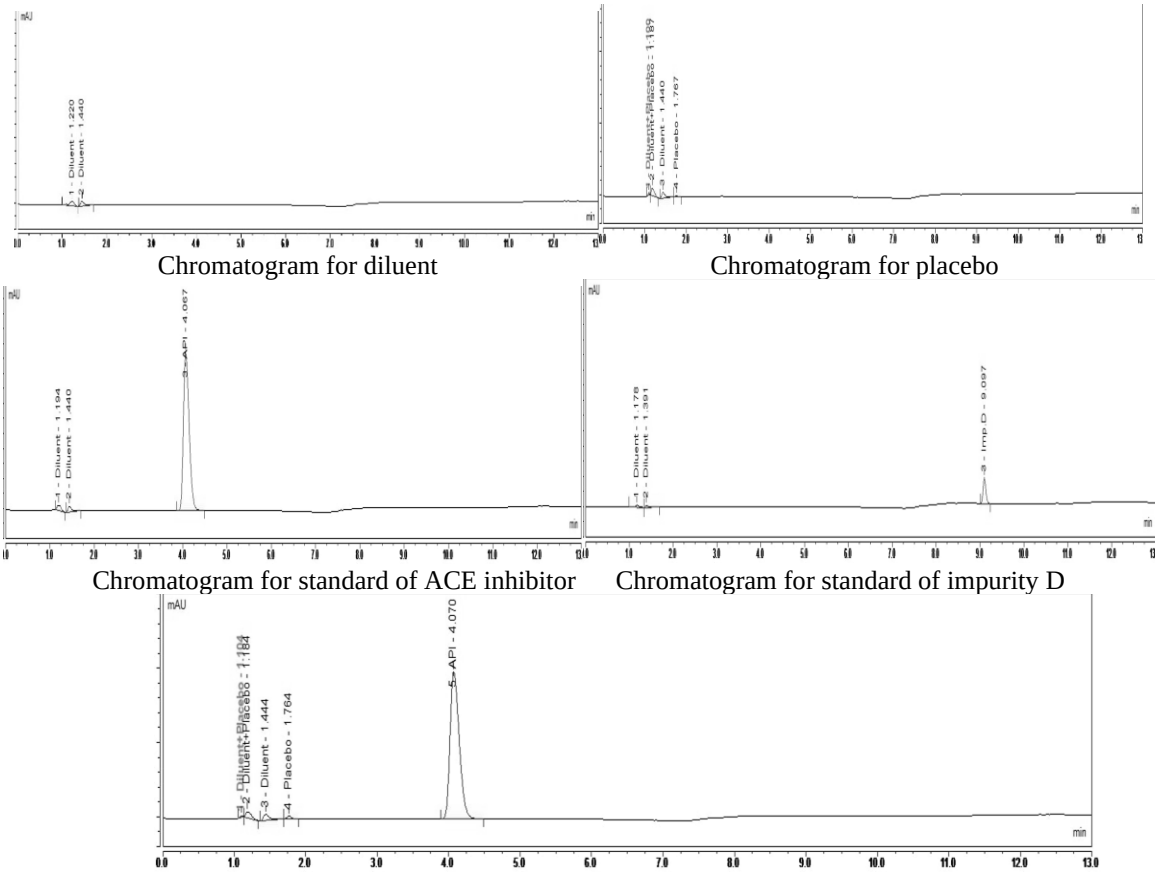

Fig. 18. Chromatograms for Specificity – Analytical method validation.

Table 14. Accuracy – Analytical method validation

Range	API calculated (mg/mL)	API found (mg/mL)	Recovery (%)	RSD (%)
50%	0.025	0.025	99.69	0.4
100%	0.051	0.051	98.83	0.4
150%	0.076	0.075	99.12	0.5
	Mean		99.21	0.4

Table 15. Linearity – Analytical method validation

Range (%)	Concentration	Area
20	0.010	3.152
40	0.020	6.257
50	0.025	7.756
80	0.040	12.506
100	0.050	15.561
120	0.060	18.591
150	0.075	23.186
Coefiicient	1.00	

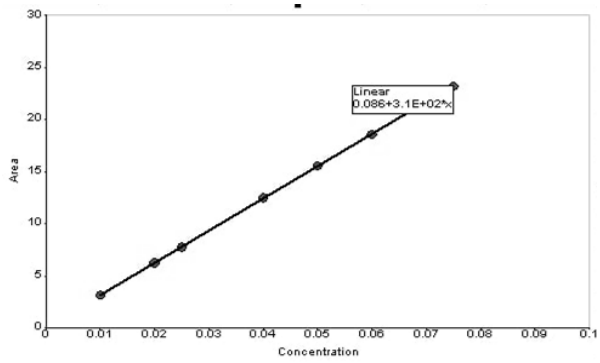

Fig. 19. Linearity Correlation Plot – Analytical method validation.

Table 15. Linearity – Analytical method validation

Range (%)	Concentration	Area
20	0.010	3.152
40	0.020	6.257
50	0.025	7.756
80	0.040	12.506
100	0.050	15.561
120	0.060	18.591
150	0.075	23.186
Coefiicient	1.00	

Table 16. Intermediate Precision – Analytical method validation

	Analyst I	Analyst II
1	97.17	98.05
2	96.86	97.10
3	96.36	97.76
4	97.01	97.62
5	98.20	98.85
6	98.24	98.59
Average (%)	97.30	97.99
Variance	0.57	0.42
F value	1.38	

Table 17. Stability of solution – Analytical method validation

	Standard		Sample	
Time interval	Area	Difference (%)	Area	Difference (%)
Fresh preparation	15.6019	/	14.9571	/
48 hours	15.7306	100.8	14.9983	100.3
72 hours	15.6417	100.3	14.9577	100.0
168 hours	15.5740	99.8	14.8883	99.5

Table 18. Filter recovery – Analytical method validation

	Area	Recovery (%)
Unfiltered	15.6144	/
Filtered	15.6641	100.3
	15.6794	100.4
	15.6571	100.3
	15.6136	100.0
	15.8820	101.7
	15.8150	101.3
	Average	100.7
	RSD	0.7

The intermediate precision of the method was determined by comparing the results from testing the sample solution by two analysts under independent conditions. Results in Table 16 represent that F-value is < 3.18, confirming the precision of the proposed method.

From data in Table 17 it is evident that the standard and sample solution are stable at least 7 days, stored at 4°C (the difference between the peak areas obtained at initial and different time interval are not more than 2.0 %).

For filter loss**,** recovery between mean unfiltered test result and each filtered test result is between 98.0% and 102.0% and RSD is not more than 2.0%. The results in Table 18 confirm that the results were not affected when the standard solution is filtered through 0.2 μm RC filter.

Conclusion

Using the Analytical quality by design approach, method for determination of content of ACE inhibitor in tablets containing 5 mg of active substance was developed and optimized. The final analytical method, is accomplished with Fusion and MODDE experimental design plans and statistical tools for estimation of obtained chromatographic responses. Influence of each method parameter and their interaction on responses of interest were properly evaluated and best chromatographic conditions were achieved.

Method is validated for specificity, linearity, accuracy, precision, robustness, stability of the solutions and filter study and found to be meeting the predetermined acceptance criteria. Hence this method can be introduced into the routine use for the determination of content of ACE inhibitor in tablets containing 5 mg of active substance.

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Резиме

Развој и оптимизација на HPLC метод за определување на содржина на ангиотензин-конвертирачки ензим во таблети со пристап на аналитички квалитет преку дизајн

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Клучни зборови: аналитички квалитет преку дизајн, развој и оптимизација на аналитички метод, валидација на метод, ангиотензин – конвертирачки ензим (АЦЕ) инхибитор

Имплементацијата на пристапот аналитички квалитет со дизајн е значаен чекор во развојот на робусен аналитички метод за секојдневна употреба низ животниот циклус на еден производ. Овој модел е одлична стратегија за развој на аналитички метод, со цел да се постигне висок степен на робусност и намален ризик од резултати кои се надвор од тренд (OOT) и надвор од спецификација (OOC), правејќи го економски исплатлив. Целта на овој труд е имплементација на софтвери кои користат експериментални дизајни како брза и ефикасна алтка за развој на метод за квантитативно определување на содржина на АСЕ инхибитор во таблети. Користејќи статистички модели во развојот и оптимизацијата на методот, влијанието на секој фактор врз одговорот од интерес е обработен и податоците се искористени да се најде оптимален хроматографски одговор. Најдобра хроматографска сепарација се постигнува за време од 13 минути, на колона Zorbax SB C18, 150 x 4,6 mm, (5 μm), со мешавина од 15 mM фосфатен пуфер, pH 1,8 и ацетонитрил како мобилна фаза, со градиентна елуција и температура на колона 60 °C. Валидираниот метод е специфичен, линеарен, точен, прецизен и робусен за определување на ангиотензин - конвертирачки ензим во таблети.