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QBD APPROACH FOR THE DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF FORMOTEROL FUMARATE AND ACLIDINIUM BROMIDE IN PRESSURIZED METER DOSE INHALER

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Keywords:

RP-HPLC, Quality by design, Aclidinium bromide, Formoterol Fumarate, Stress studies

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ABSTRACT: A Quality design approach to method development involves method goal identification, method scouting and evaluation, method selection, and risk assessment. The present study describes the risk-based HPLC method development and validation of Formoterol fumarate and Aclidinium bromide in a pharmaceutical dosage form. The chromatographic conditions were optimized with the Design Expert software 11.0 version *i.e.*, Kromasil C18 (250×4.6 mm, 5 µm) Mobile phase used was 0.01N Kh2po4 (pH :3.0) (52.4%): Acetonitrile (47.6%), flow rate was found to be 1.0ml/min with retention times of formoterol 2.456 min and Aclidinium 3.573min. The developed method was found to be linear in the range of Formoterol 1.5-9µg/ml, aclidinium 50-300µg/ml with a correlation coefficient of 0.999 for both drugs. The % RSD of intraday and inter-day precision for Formoterol 0.3 & 1.0, Aclidinium 0.7 & 0.4. The robustness values were less than 2%.The assay was found to be 99.91%&99.99% for formoterol and aclidinium bromide. The method validation parameters were in the prescribed limit as per ICH guidelines. Stress studies reveal that both drugs were degraded more in acidic conditions than in alkaline, neutral, oxidative, photolytic and thermal conditions. Hence, the proposed method was stability indicating, using QbD approach all the method parameters were better understood that, reduces the time and cost of the analysis.

INTRODUCTION: Formoterol fumarate, (*N*-[2-hydroxy-5-[(1 R)-1-hydroxy-2-[[(2 R)-1-(4-methoxyphenyl) propan-2-yl] amino] ethyl] phenyl] formamide [9] is a long-acting β_2-adrenoceptor agonist. It was proven to be a very effective broncho-dilating agent in the treatment of nocturnal and exercise-induced asthma. Inhaled formoterol fumarate acts locally in the lung as a bronchodilator.

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To stimulate intracellular adenyl cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3', 5'-adenosine monophosphate (cyclic AMP). An increased cyclic AMP level causes relaxation of bronchial smooth muscle and inhibits the release of pro-inflammatory mast-cell mediators such as histamine and leukotrienes.

The structure of Formoterol fumarate is shown in **Fig. 1.** Aclidinium bromide (3R)-3-{[hydroxy-2,2-bis(thiophen - 2 - yl) acetyl] oxy} - 1 - (3 phenoxypropyl) - 1 – azabi – cyclo [2.2.2] octan-1-ylium bromide [10] is an anticholinergic for the long-term management of chronic obstructive pulmonary disease (COPD). It was slightly soluble

in water, soluble in methanol, and very soluble in acetonitrile. Aclidinium is a long-acting, competitive, and reversible anticholinergic drug specific to the acetylcholine muscarinic receptors. The structure of Aclidinium bromide is shown in **Fig. 2.**

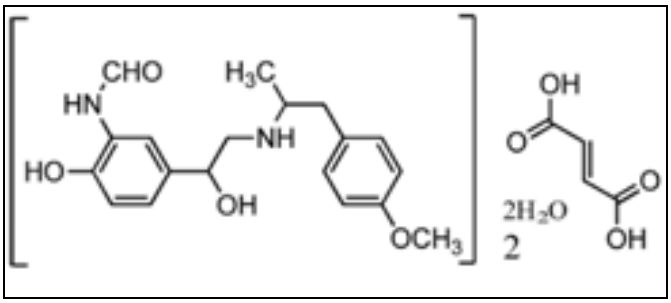


FIG. 1: STRUCTURE OF FORMOTEROL FUMARATE

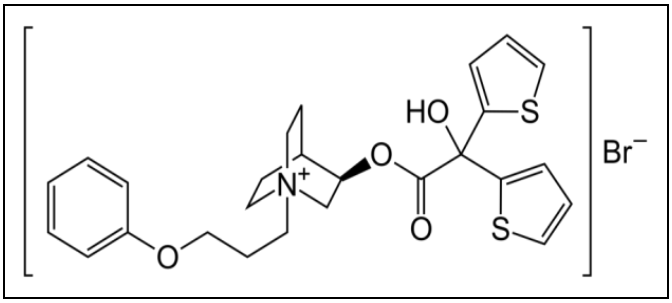


FIG. 2: STRUCTURE OF ACLIDINIUM BROMIDE

The literature survey reveals that very few analytical methods like HPLC [11-14] and impurity profiling [15] were reported to estimate these drugs in individual and combined dosage forms. Hence, the authors attempted to develop a stability-indicating RP-HPLC method for simultaneously estimating two drugs in a pressurized meter dose inhaler using the QbD approach. Applying QbD principles during analytical method development and validation 1-7 leads to a reduction in the number of trials, cost, and time.

MATERIALS AND METHODS:

Chemicals and Reagents: Working standards of Formoterol Fumarate (99.85%) and Aclidinium bromide (99.93%) were procured from Spectrum pharma pvt ltd (Hyderabad). Hydrochloric acid (AR) and sodium hydroxide (AR) were obtained from Merck India Pvt Ltd. Hydrogen Peroxide (AR) was purchased from Qauligens. Orthophosphoric acid (AR) and potassium dihydrogen orthophosphate (AR) were obtained from S.D. Fine chem Ltd. HPLC grade Acetonitrile and methanol were purchased from Fischer scientific. HPLC grade water used throughout analysis was obtained from the Merck milli-Q water purification unit.

Apparatus and Equipment: HPLC studies were carried out on WATERS HPLC 2965 SYSTEM with Photo diode array detector (PDA) set at 234 nm for uv detection. viz; Kromasil C18 (150×4.6 mm, 5µm) , Azilent C18 (150×4.6 mm, 5µm), Altima C18 (150×4.6 mm, 5µm) and ODS C18 (150×4.6 mm, 5µm) columns were utilized in the study. Design Expert® (11.0) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was used for generation of contour plots and 3D space. Entire stress studies were carried out on radley apparatus having continuous stirring and temperature adjustable knob facility. Stress samples were preserved at -30°C freezer facility (Thermo scientific) pH meter (Eutech instruments, India) was used to check the pH of all solutions. Other equipment sonicator (ePEI ultrasonic generator), Analytical balance (Mettler Toledo), vortex meter (IKA Vortex), Hot air oven (Yorco scientific) and received drug samples were authenticated by melting point apparatus (BUCHI), FT-IR/ATR (BRUKER ALFA), UV-VIS spectrophotometer (Shimadzu-1800, japan).

Calibration of Instruments and Apparatus: Calibration of instruments like HPLC, pH meter and the weighing balance was done. HPLC was calibrated for Flow rate accuracy, gradient performance check, injector precision & linearity, Detector linearity, Wavelength Accuracy and Carry over. pH meter was calibrated by the triple point method.

Identification of Drug Sample:

By UV-VIS Spectra: 10µg/mL concentration of Formoterol Fumarate and Aclidinium bromide were prepared using methanol and UV spectrum was recorded.

By IR Spectra: Formoterol Fumarate and Aclidinium bromide were scanned in FT-IR spectrometer (Bruker-ALFA) from 4000 to 400 cm^{-1} and characteristic peaks of functional groups were identified.

By Mass Spectra: Mass of Formoterol Fumarate and Aclidinium bromide were determined using

LC-MS/MS (Q-ToF) by injecting dilute drug samples in methanol.

By Melting Point Apparatus: Formoterol Fumarate and Aclidinium bromide were subjected to melt in programmable melting point apparatus (BUCHI) and melting point value was compared with the reported value.

Method Development:

Preparation of Drug Solution: The stock solution was prepared by dissolving 3mg of Formoterol Fumarate and 100mg of Aclidinium bromide in 50ml clean, dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and making up to the final volume with diluents. Working solutions of different concentrations were prepared by withdrawing the appropriate volume of solution from the stock solution.

Preparation of Buffer:

0.01N Potassium Dihydrogen Ortho Phosphate: Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask, add about 900ml of milli-Q water and degas to sonicate and finally make up the volume with milli-Q water then added 1ml of Triethylamine then pH adjusted to 3.0 with dil. Orthophosphoric acid solution.

0.1% Ortho Phosphoric Acid Buffer: 1ML of Ortho phosphoric acid solution in a 1000ml of the volumetric flask; add about 100ml of milli-Q water, and the final volume makes up to 1000 ml with milli-Q water

Initial Chromatographic Conditions: Initial HPLC runs of Formoterol Fumarate and Aclidinium bromide with10 µg/mL concentration were performed using.

- Different buffer *viz*, Potassium dihydrogen ortho phosphate and Ortho phosphoric acid.
- Different organic modifier *viz*, acetonitrile and methanol
- Different columns such as Kromasil C18 (250×4.6 mm,5µm), Azilent C18 (150×4.6 mm, 5µm), Altima C18 (150×4.6 mm,5µm) and Phenomenex C18 (150×4.6 mm, 5µm) column.

Optimization of Method: The method was optimized using central composite design (CCD). The initial trials were needed to optimize the final method. A total of three factors viz; % Organic concentration, flow rate, and Column temperature were needed to be optimized.

So, CCD was used to optimize these parameters, which were varied over three levels (high, medium, and low) different levels of three parameters ranging from 0.83-1.17ml/min flow rate, 46.59-53.41% potassium dihydrogen orthophosphate (pH-3), Column temperature ranging from 24.95°C-35.05°C respectively were taken and counter, 3D surface plot showing the effect of each parameter on Retention Time, Resolution, Asymmetry and Theoretical plates (CQA) were generated.

A desirability function is applied to the optimized conditions to predict retention time, resolution, asymmetry, and theoretical plates. Analysis of Variance was also calculated.

Designing of Forced Degradation Studies:

Acid Hydrolysis: To 1 ml of working standard solutions of both drugs, add 1 ml of 2N HCl solution and keep in the Radley apparatus with continuous stirring at 70ºC for 90 min. These stressed samples were neutralized to pH 7, with 2N NaOH, and diluted, analyzed by the HPLC system.

Base Hydrolysis: To 1 ml of working standard solution of both the drugs, add 1 ml of 2N NaOH solution and kept in Radley apparatus with continuous stirring at 70º C for 90 min. These stressed samples were neutralized to pH 7 with 2N HCL and diluted, analysed by the HPLC system.

Neutral Hydrolysis: To 1ml of working standard solution of both the drugs, add 1ml of water and keep in Radley apparatus with continuous stirring at 70º C for 90 min. These stressed samples were diluted and analysed by the HPLC system.

Oxidative Study: To 1 ml of working standard solution of both drugs, add 1 ml of 3% H_2O_2 solution, and the samples were kept in a dark area without disturbance at room temperature for 24 h. These stressed samples were diluted and analyzed by the HPLC system.

Thermal Degradation: 3mg of Formoterol Fumarate and 100mg of Aclidinium bromide were transferred into a Petri dish and kept in hot air oven

at 70ºC for 24 hrs. Sampling was done atmultiple time points. Samples were dissolved in a diluent, and made suitable dilutions, analyzed by the HPLC system

Photo Degradation: 3mg of Formoterol Fumarate and 100mg of Aclidinium bromide were uniformly spread in a Petri dish and exposed to direct sunlight for 8 hrs. Sampling was done at multiple time points and analyzed by the HPLC system.

Method Validation: The final optimized chromatographic analytical method was validated as per ICHQ2(R1) guidelines for system suitability, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

Linearity: Standard calibration curves were generated with six different concentrations, including the LOQ by making serial volume-to-volume dilution of stock solution I over the range of 1.5-9µg/ml of Formoterol Fumarate and 50-300µg/ml of Aclidinium bromide. Linear calibration curves were generated between the average peak area and drug concentration. The linearity was examined using linear regression, which was calculated by the least square regression method. The results obtained were as shown in **Table 9.**

Precision: The precision of the proposed analytical method was determined by repeatability (intraday) and intermediate precision (inter-day). Repeatability defines the use of the analytical procedure within a laboratory over a short time that was examined by assaying the samples during the same day. Intermediate precision was evaluated by comparing the assays on different days. SD and %RSD were determined. System precision also performed. The results obtained were as shown in **Tables 10** & **11.**

Accuracy: The accuracy experiments were carried out using the standard addition method. Three different recovery level concentrations (50%, 100%, and 150%) of standards were added to pre-analyzed samples in triplicate. The percentage recovery of Formoterol Fumarate and Aclidinium bromide at each level in triplicate were calculated, and the mean percentage recovery (n=9) and the relative standard deviation were determined. The results obtained were as shown in **Tables 12** & **13.**

Limits of Detection and Quantitation: Limits of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. The detection limit was referred to as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was referred to as the lowest concentration level, providing a peak area with a signal-to-noise ratio higher than ten. The results obtained were as shown in **Table 14.**

System Suitability: The system suitability was determined by taking six replicates of both the drugs at concentrations of Formoterol fumarate 3µg/ml and aclidinium bromide 100µg/ml. The acceptance criteria was ± 2% for the percent coefficient of variation (% CV) for the peak area, retention time of drug, USP Plate Count and asymmetry.

Robustness: Robustness is one of the validation parameter; it is a measure of the method's capacity to remain unaffected by small, deliberate changes in chromatographic conditions was studied by testing the influence of small changes in the organic content of mobile phase (±2%), flow rate (±2%) and Temp (±°C). The results obtained were as shown in **Table 15.**

RESULTS AND DISCUSSION:

Selection of Detection Wavelength: After scanning from 200 to 400nm in a UV-VIS spectrophotometer, Formoterol Fumarate showed absorption maxima at 225nm in methanol, and Aclidinium bromide showed absorption maxima at 230 nm in methanol. Isobestic points for both drugs were determined.

Method Development by Doe:

Parameter Selection: Various preliminary HPLC trials were carried out for a selection of column and organic modifiers. The choice of a Kromasil C18 column based on the preliminary investigation was selected among Azilent C18, Altima C18, phenomenex c18 column, and Kromasil C18 (250×4.6 mm, 5 µm) columns. A Kromasil C18 column has less tailing, higher theoretical plate and good shape of drugs peaks compared to the phenomenex column. Selection of a suitable organic modifier was also important to get better selectivity with adequate separation of all analytes.

Commonly used organic solvents for the reversed-phase HPLC include Methanoland Acetonitrile. From that trial, acetonitrile was an ideal and suitable organic modifier compared to methanol because Formoterol Fumarate and Aclidinium bromide were solubilized in acetonitrile compared to methanol. Therefore, acetonitrile was selected and finalized as the organic modifier for further optimization study.

Optimization of Proposed Method: To understand the results, 2D contour plots and 3D plot were generated after processing all data using the Design Expert® software **Fig. 3A** and **3B**.

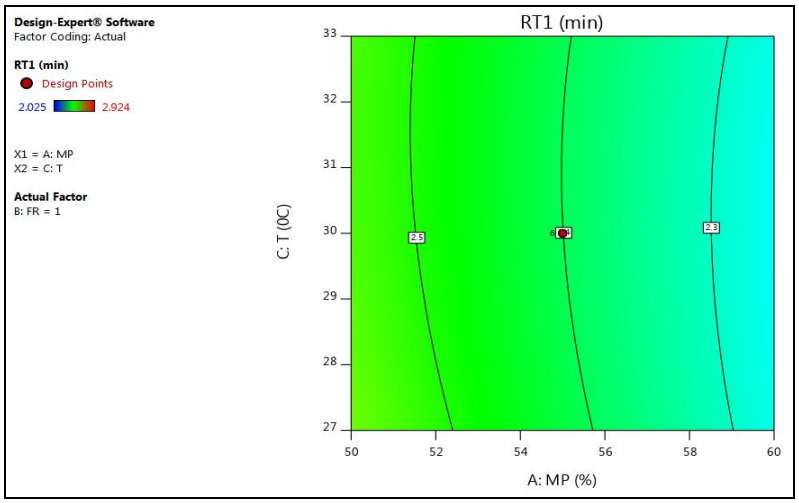
It shows the two-dimensional contour plot as a function of % organic concentration, flow rate, and column temperature. Based on the color code, the working region can be easily identified. Retention time maps represent the value of the retention time, with warm "red" colors indicating larger retention time, cold "blue" colors lower, and light green to a yellow color representing intermediate retention time. The results of the ANOVA test for retention times **Tables 2** & **3**, resolution **Table 4**, Asymmetry **Table 5** and Theoretical plates **Table 6** for both drugs were mentioned.

TABLE 1: DESIGN SUMMARY OF CCD

Design Summary					
Study Type: Response surface, Design Type: Central composite Design, Design Model: Quadratic			ATP: Robustness, CQA: Retention time, Theoretical plates and Asymmetry, Runs: 20		
CMPs	Unit	Type	Subtype	Min.	Max.
Column temperature	℃	Numeric	Continuous	24.95	35.05
Flow rate	ml/min	Numeric	Continuous	0.83	1.17
% Org ratio	%v/v	Numeric	Continuous	46.59	53.41

TABLE 2: ANOVA TABLE FOR RETENTION TIME OF FORMOTEROL FUMARATE USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	Response
Model	1.31	9	0.1460	79.85	< 0.0001	Significant
A-MP	0.2792	1	0.2792	152.68	< 0.0001	
B-FR	1.00	1	1.00	548.58	< 0.0001	
C-T	0.0009	1	0.0009	0.4806	0.5040	
AB	0.0009	1	0.0009	0.5174	0.4884	
AC	0.0005	1	0.0005	0.2713	0.6138	
BC	0.0002	1	0.0002	0.0936	0.7659	
A^2	7.217E-07	1	7.217E-07	0.0004	0.9845	
B^2	0.0278	1	0.0278	15.18	0.0030	
C^2	0.0025	1	0.0025	1.36	0.2709	
Residual	0.0183	10	0.0018			
Lack of Fit	0.0181	5	0.0036	109.48	< 0.0001	significant
Pure Error	0.0002	5	0.0000			
Cor Total	1.33	19				



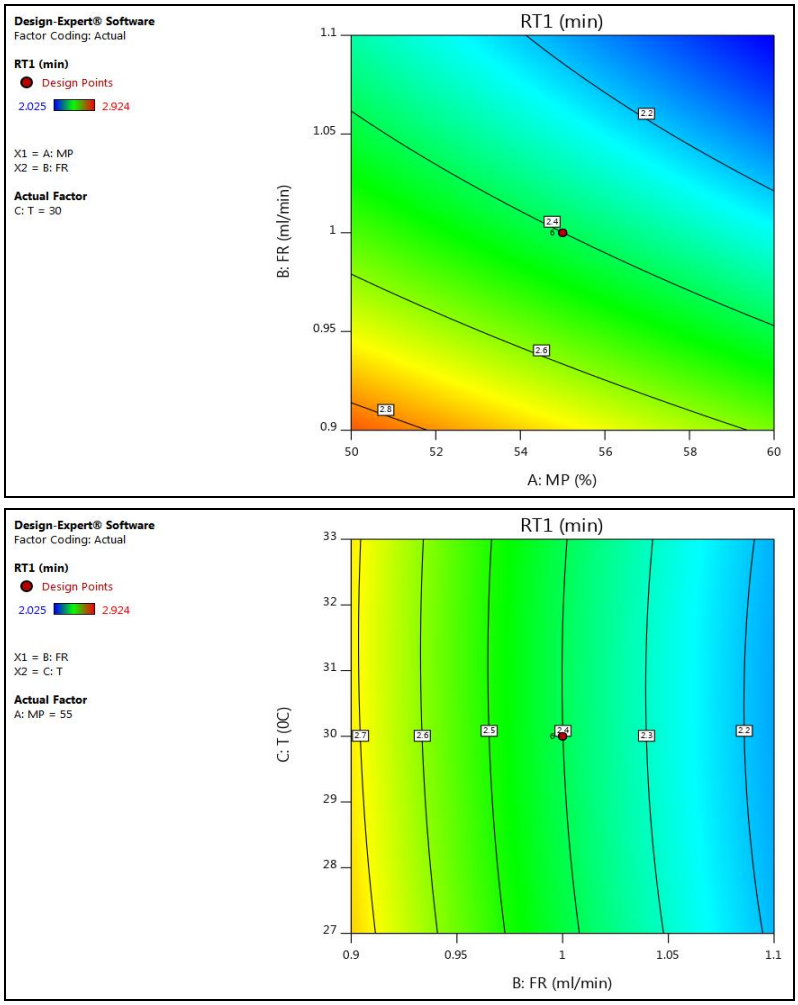
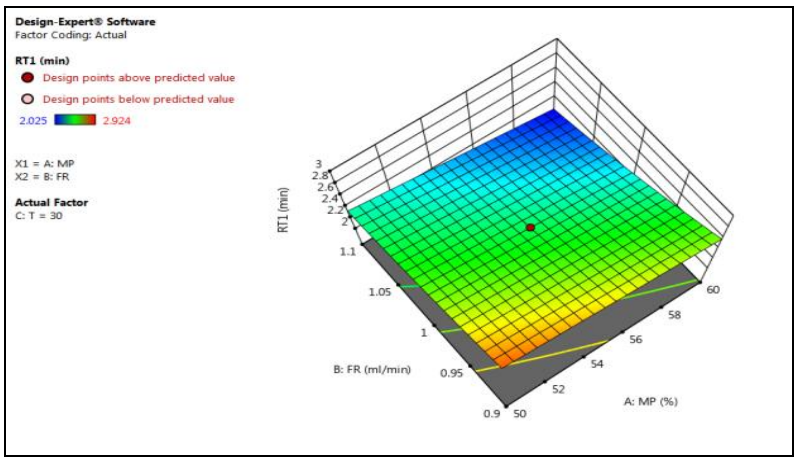


FIG. 3(A): 2D CONTOUR PLOTS OF RETENTION TIME AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

In order to understand the results, 2D contour plots and 3D plot were generated after processing all data using the Design Expert® software **Fig. 3A** and **3B**. It shows the two-dimensional contour plot as a function of % organic concentration, flow rate, and column temperature. Based on the color code, the working region can be easily identified. Retention time maps represent the retention time value, with warm "red" colors indicating larger retention time, cold "blue" colors lower, and light green to a yellow color representing intermediate retention time. The results of the ANOVA test for retention times **Tables 2** & **3**, resolution **Table 4**, Asymmetry **Table 5,** and Theoretical plates **Table 6** for both drugs were mentioned.



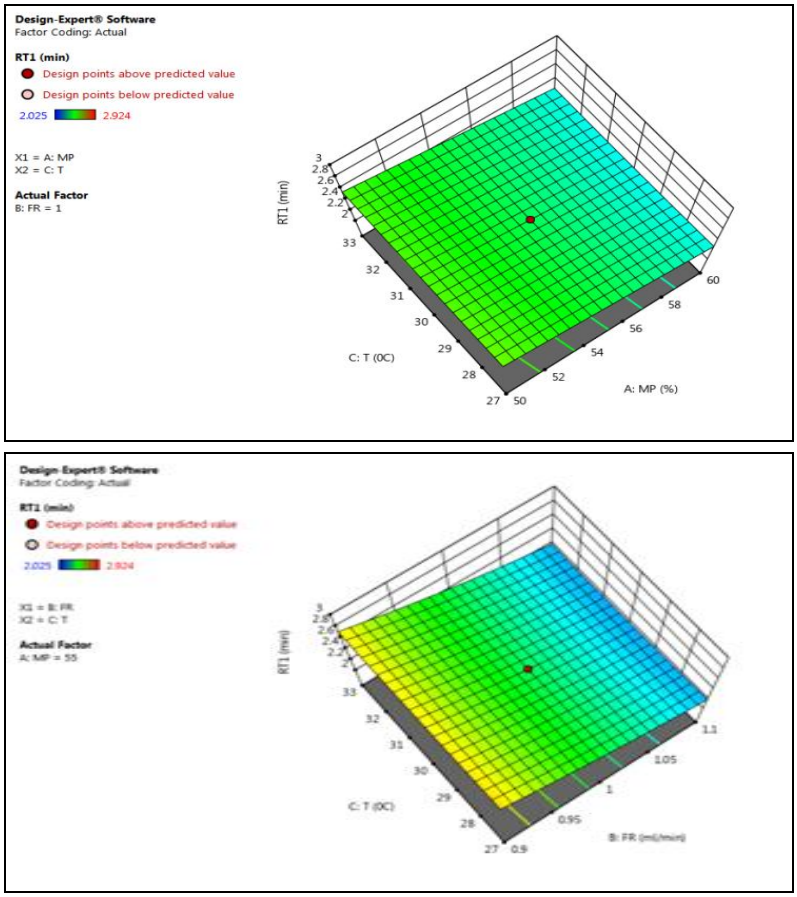
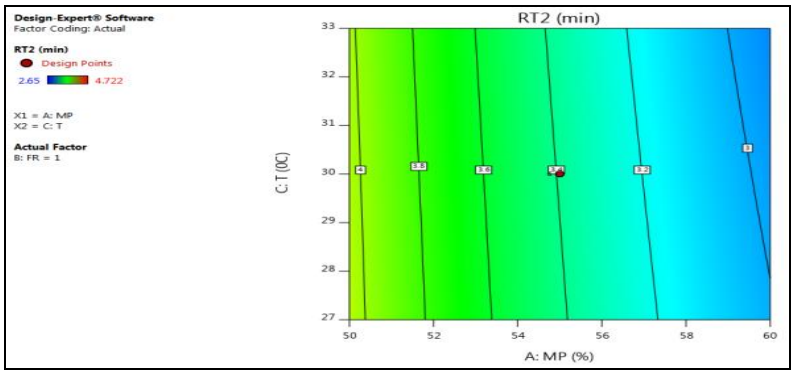


FIG. 3(B): 3D CONTOUR PLOTS OF RETENTION TIME AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

TABLE 3: ANOVA TABLE FOR RETENTION TIME OF ACLIDINIUM BROMIDE USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	Response
Model	6.10	9	0.6778	141.47	< 0.0001	significant
A-MP	3.90	1	3.90	813.07	< 0.0001	
B-FR	1.98	1	1.98	412.47	< 0.0001	
C-T	0.0111	1	0.0111	2.32	0.1588	
AB	0.0031	1	0.0031	0.6431	0.4412	
AC	0.0010	1	0.0010	0.2161	0.6520	
BC	0.0112	1	0.0112	2.33	0.1577	
A^2	0.1892	1	0.1892	39.50	< 0.0001	
B^2	0.0201	1	0.0201	4.19	0.0677	
C^2	0.0000	1	0.0000	0.0031	0.9565	
Residual	0.0479	10	0.0048			
Lack of Fit	0.0477	5	0.0095	187.00	< 0.0001	significant
Pure Error	0.0003	5	0.0001			
Cor Total	6.15	19				



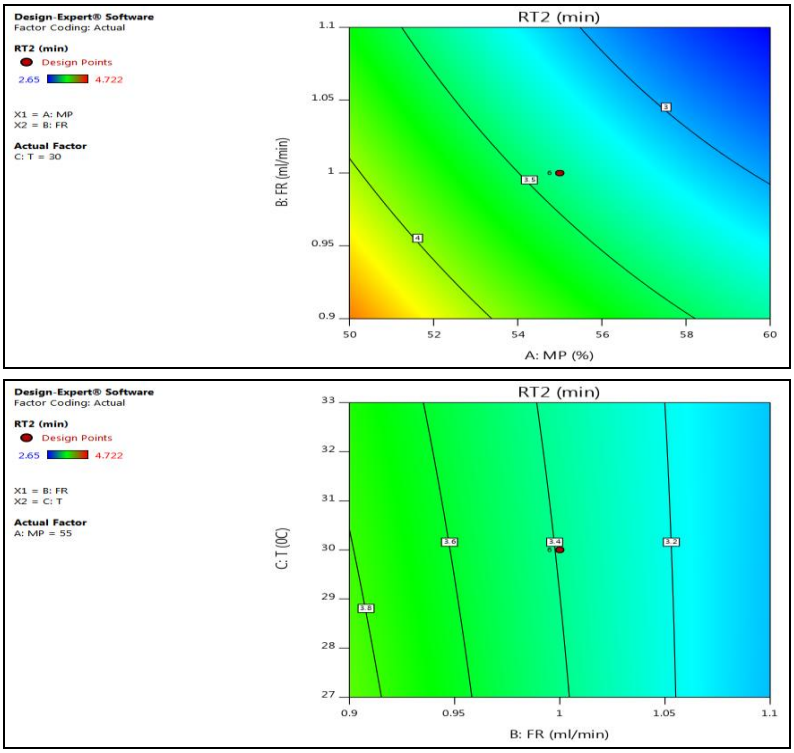
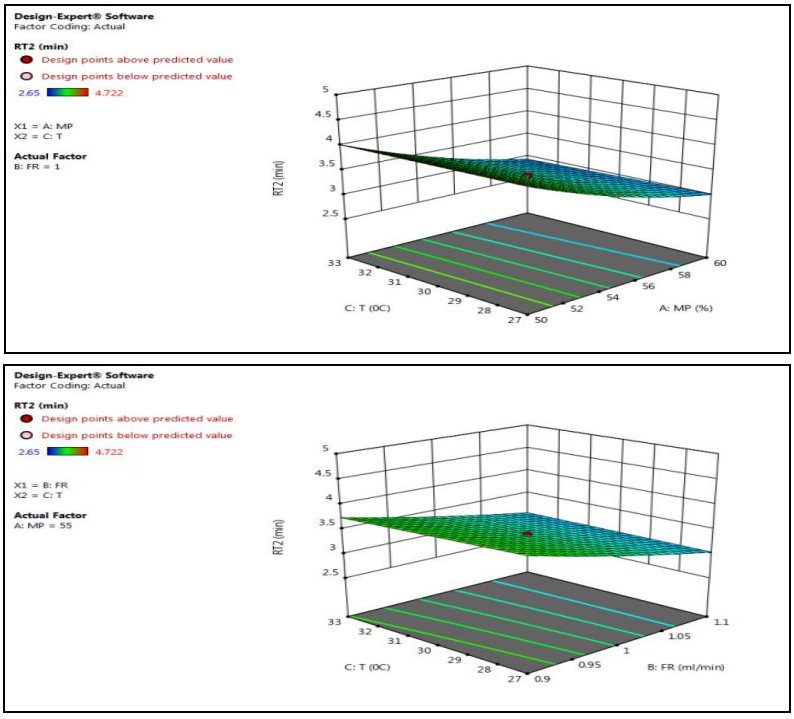


FIG. 4(A): 2D CONTOUR PLOTS OF RETENTION TIME AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

In order to understand the results, contour plots and 3D plot were generated after processing all data using the Design Expert® software **Fig. 4A** and **4B**. It shows the two-dimensional contour plot as a function of % organic concentration, flow rate, and column temperature. Based on the color code, the working region can be easily identified. Retention time maps represent the retention time value, with warm "red" colors indicating larger retention time, cold "blue" colors lower, and light green to a yellow color representing intermediate retention time. 2D and 3D counterplots for resolution **Fig. 5A** and **5B**, Asymmetry **Fig. 6A** and **6B** & Theoretical plates **Fig. 7A** and **7B** were also plotted using Design Expert® software.



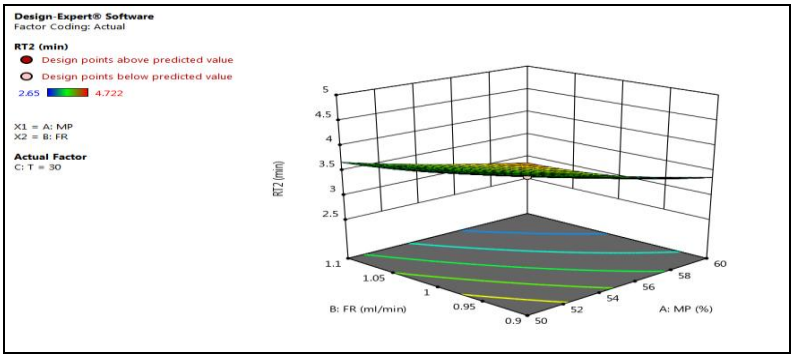
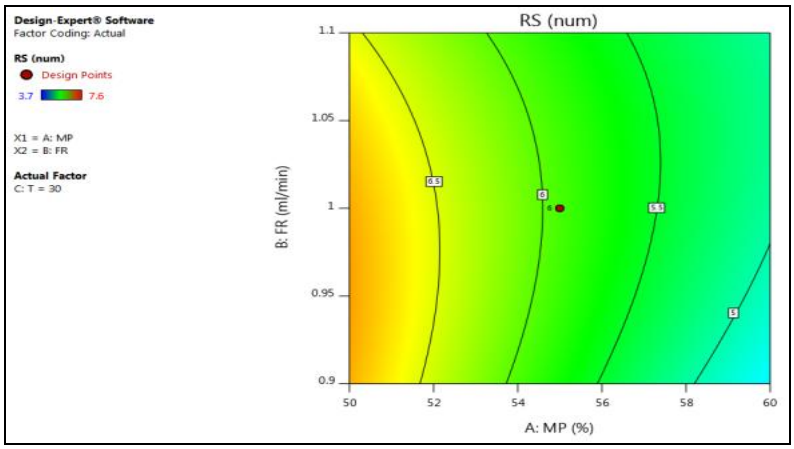
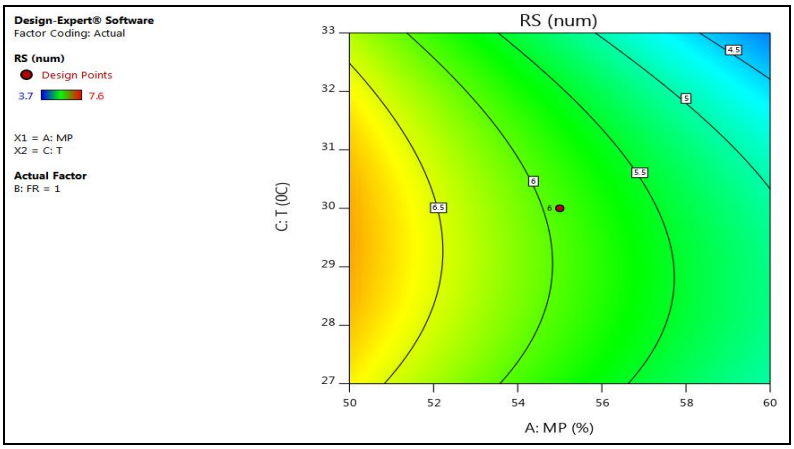


FIG. 4(B): 3D CONTOUR PLOTS OF RETENTION TIME AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

TABLE 4: ANOVA TABLE FOR RESOLUTION USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	Response
Model	17.51	9	1.95	6.74	0.0031	significant
A-MP	12.28	1	12.28	42.54	< 0.0001	
B-FR	0.0025	1	0.0025	0.0088	0.9271	
C-T	1.15	1	1.15	3.99	0.0735	
AB	0.3200	1	0.3200	1.11	0.3171	
AC	0.1250	1	0.1250	0.4331	0.5253	
BC	0.0200	1	0.0200	0.0693	0.7977	
A^2	0.0855	1	0.0855	0.2964	0.5981	
B^2	0.6104	1	0.6104	2.11	0.1765	
C^2	2.96	1	2.96	10.26	0.0094	
Residual	2.89	10	0.2886			
Lack of Fit	2.87	5	0.5746	215.47	< 0.0001	significant
Pure Error	0.0133	5	0.0027			
Cor Total	20.40	19				





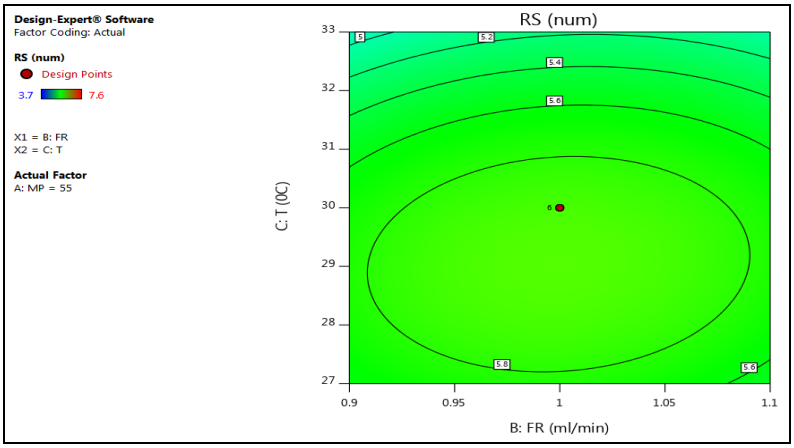


FIG. 5(A): 2D CONTOUR PLOTS OF RESOLUTION AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

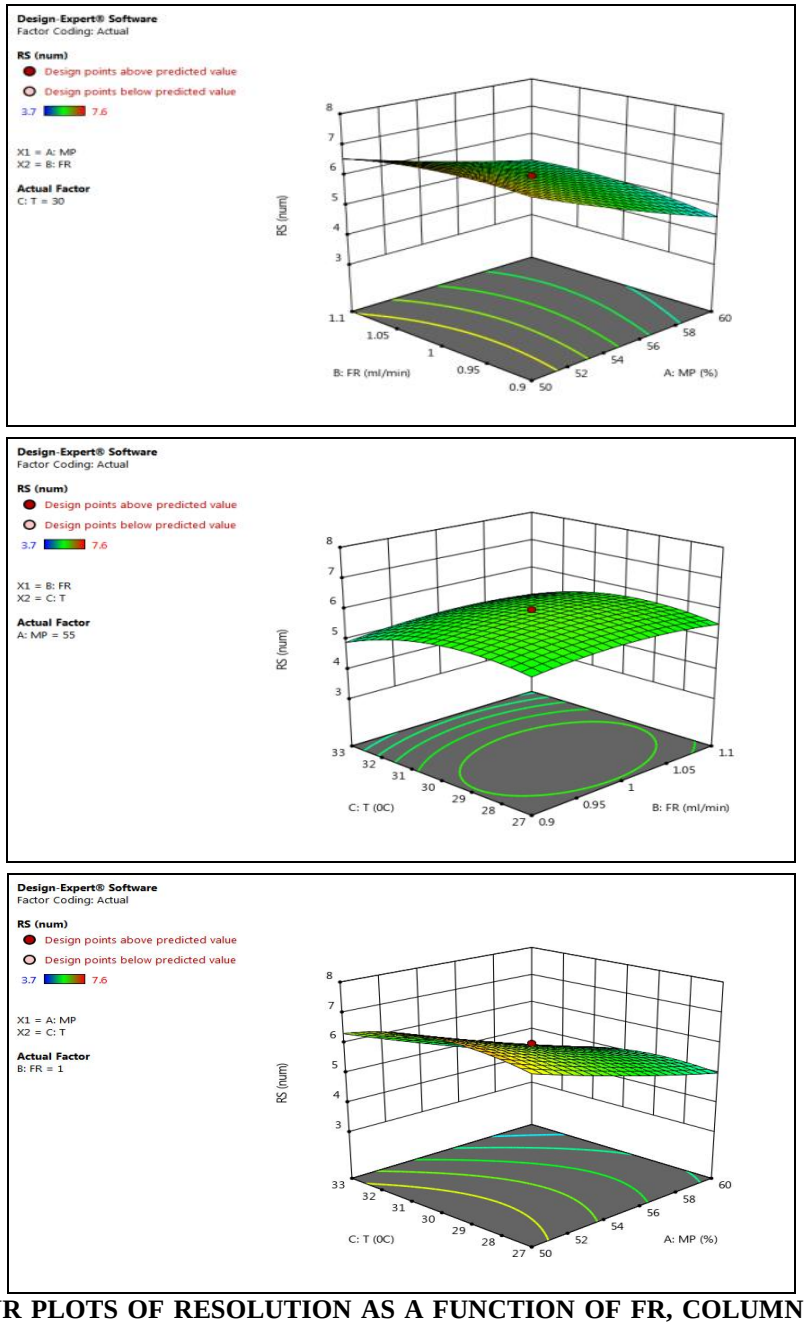


FIG. 5(B): 3D CONTOUR PLOTS OF RESOLUTION AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

TABLE 5: ANOVA TABLE FOR ASYMMETRY OF FORMOTEROL FUMARATE USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	Response
Model	0.0907	6	0.0151	2.92	0.0497	significant
A-MP	0.0297	1	0.0297	5.73	0.0325	
B-FR	0.0066	1	0.0066	1.27	0.2795	
C-T	0.0007	1	0.0007	0.1415	0.7129	
AB	0.0113	1	0.0113	2.17	0.1642	
AC	0.0312	1	0.0312	6.04	0.0288	
BC	0.0113	1	0.0113	2.17	0.1642	
Residual	0.0673	13	0.0052			
Lack of Fit	0.0589	8	0.0074	4.42	0.0592	not significant
Pure Error	0.0083	5	0.0017			
Cor Total	0.1580	19				

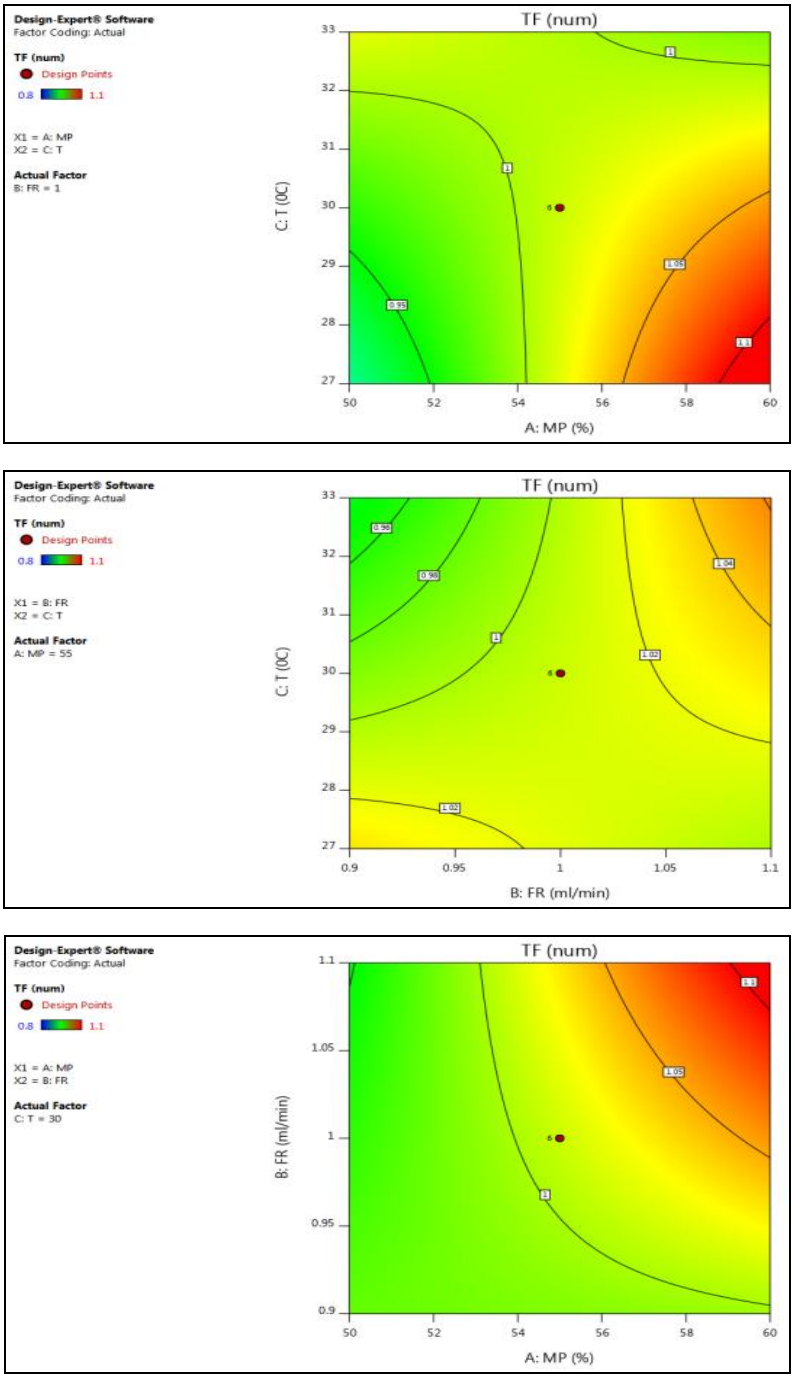


FIG. 6(A): 2D CONTOUR PLOTS OF ASYMMETRY OF FORMOTEROL FUMARATE AS A FUNCTION OF FLOW RATE, COLUMN TEMPERATURE AND ORGANIC RATIO

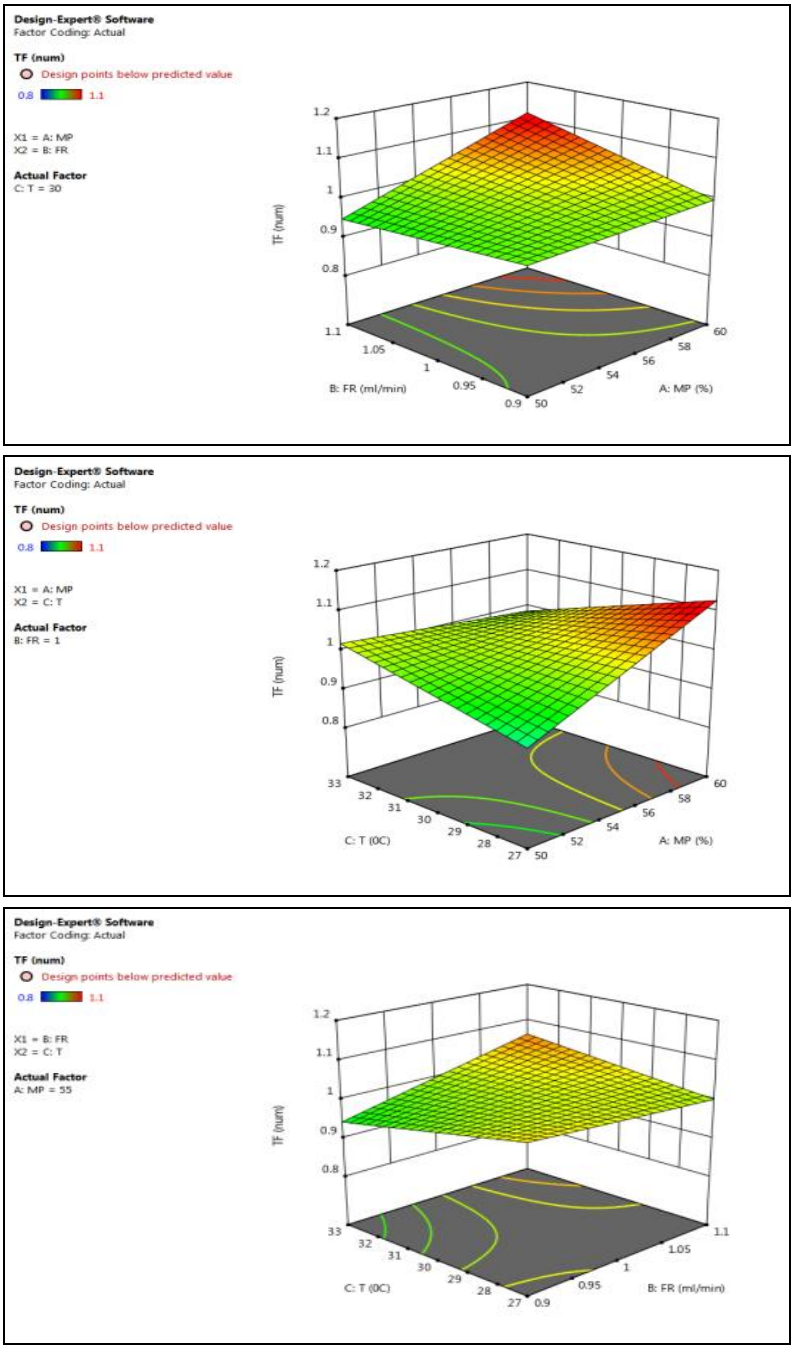


FIG. 6(B): 3D CONTOUR PLOTS OF ASYMMETRY OF FORMOTEROL FUMARATE AS A FUNCTION OF FLOW RATE , COLUMN TEMPERATURE AND ORGANIC RATIO

TABLE 6: ANOVA TABLE FOR THEORETICAL PLATES OF ACLIDINIUM BROMIDE USING CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	Response
Model	1.571E+07	9	1.746E+06	6.76	0.0031	significant
A-MP	86757.33	1	86757.33	0.3357	0.5751	
B-FR	7.037E+05	1	7.037E+05	2.72	0.1299	
C-T	5.164E+05	1	5.164E+05	2.00	0.1878	
AB	2.552E+06	1	2.552E+06	9.88	0.0105	
AC	3.114E+05	1	3.114E+05	1.20	0.2981	
BC	1.752E+06	1	1.752E+06	6.78	0.0263	
A^2	1.450E+06	1	1.450E+06	5.61	0.0393	
B^2	1.684E+06	1	1.684E+06	6.52	0.0287	
C^2	8.046E+06	1	8.046E+06	31.14	0.0002	
Residual	2.584E+06	10	2.584E+05			

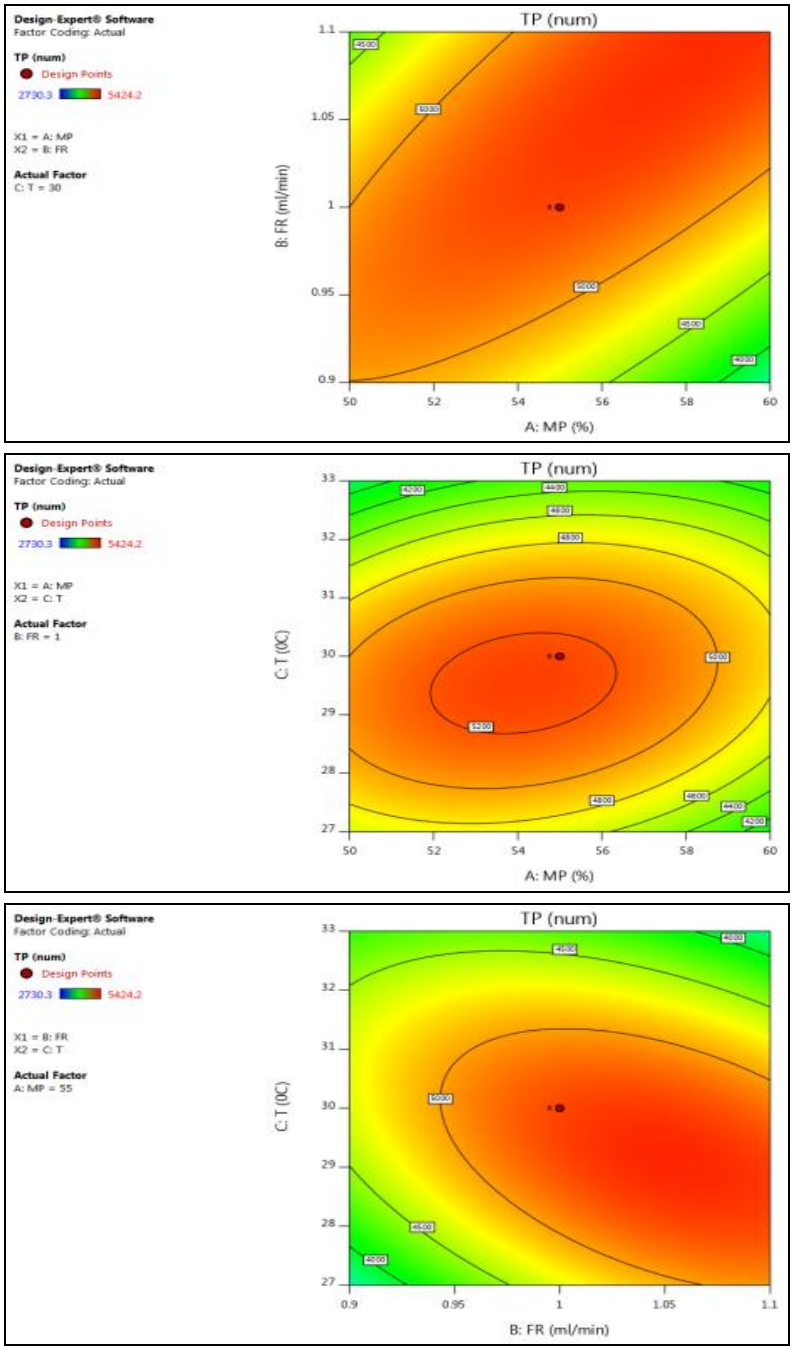
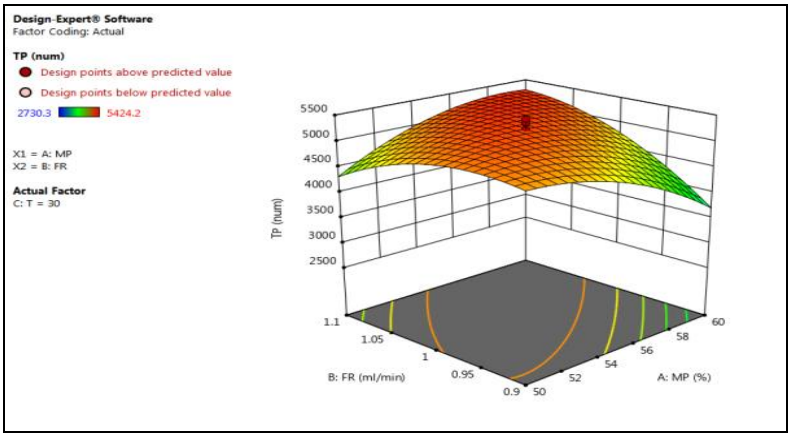


FIG. 7(A): 2D CONTOUR PLOTS OF THEORETICAL PLATES OF ACLIDINIUM BROMIDE AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO



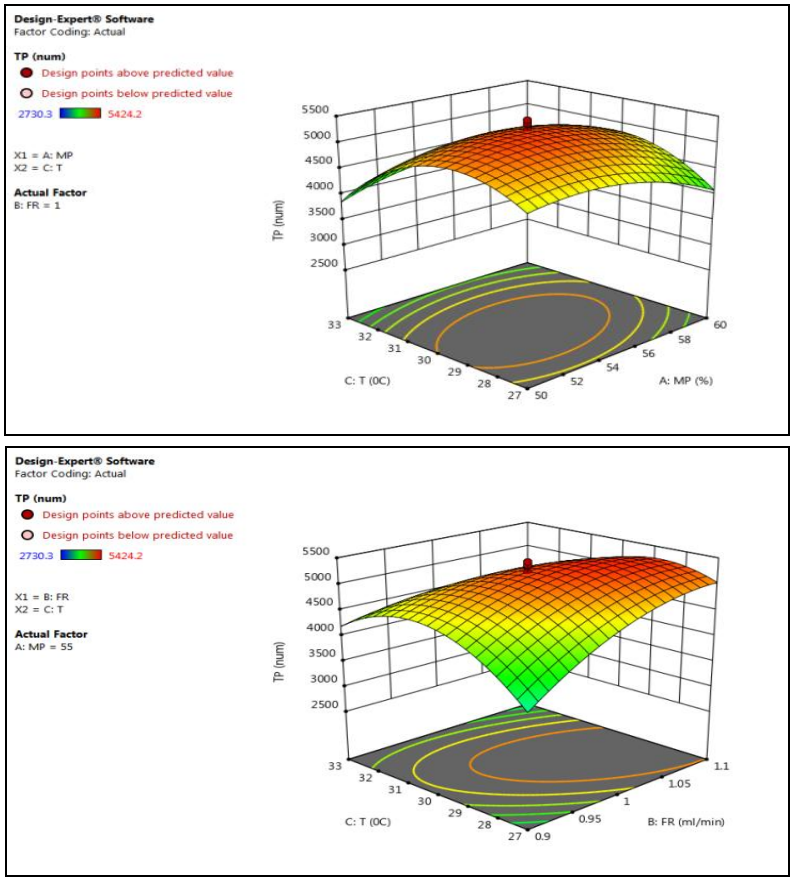


FIG. 7(B): 3D CONTOUR PLOTS OF THEORETICAL PLATES OF ACLIDINIUM BROMIDE AS A FUNCTION OF FR, COLUMN TEMPERATURE AND ORGANIC RATIO

Optimized HPLC Conditions: 3D space was obtained after processing all data using the software to understand the results. A composite desirability was applied to get an optimum set of conditions based on each response's specified goals and boundaries.

This desirability function was depends on a scale of desirability function ranges between d = 0, for a completely undesirable response, to d = 1 for a fully desirable response Based on the specified goals and boundaries for the retention time of Aclidinium bromide, Formoterol Fumarate, resolution, Asymmetry and composite desirability (D) of 1 was obtained, which gave the optimal flow rate of 1 ml/min.

To confirm this optimum set of conditions, three replicate injections of 100 µg/ml of Aclidinium bromide, and 3µg/ml of Formoterol Fumarate were analyzed to determine their observed retention time of both drugs, resolution, asymmetry and theoretical plates were within the predicted ranges. It was observed that the differences between the observed and predicted peak responses were less than 5%. The overall desirability of the final method and optimized chromatogram were shown in **Fig. 8** & **9**. By considering all these parameters, a simple and robust method was optimized. The optimized chromatographic conditions were as shown in **Table 7.**

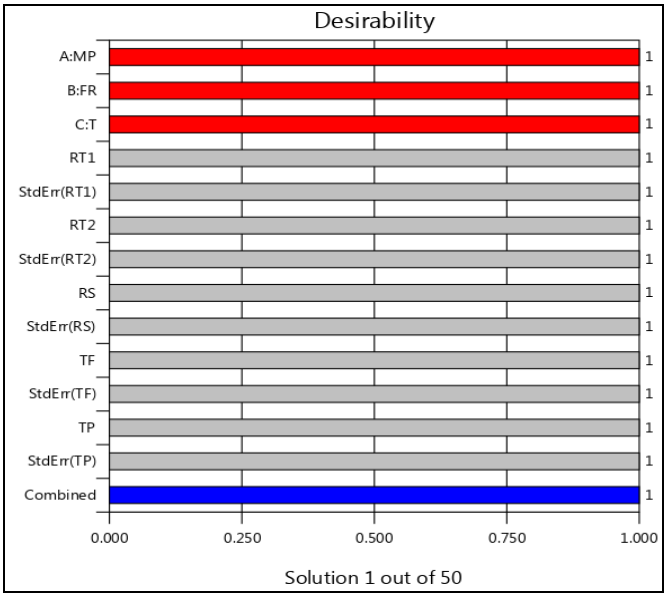


FIG. 8: OVERALL DESIRABILITY OF THE FINAL METHOD

TABLE 7: OPTIMIZED HPLC METHOD PARAMETER

Condition	Result
Mobile phase	(52.4%) 0.01N Kh2po4: Acetonitrile (47.6%)
Column	Kromasil C18 (250×4.6 mm, 5 µm)
pH	3.0
Flow rate	1ml/min
Column temperature	27.5°C

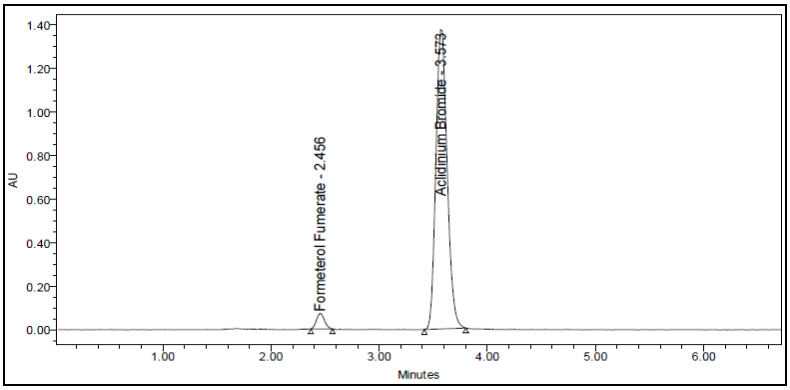


FIG. 9: A CHROMATOGRAM OF THE FINAL OPTIMIZED METHOD

Stress Degradation Studies:

Acid Hydrolysis: Both the drugs were exposed to 2N HCl, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 90 mins, it showed that 6.34% of Aclidinium bromide and 6.11% of Formoterol Fumarate degradation in acid hydrolysis. The obtained chromatogram as shown in **Fig. 10.**

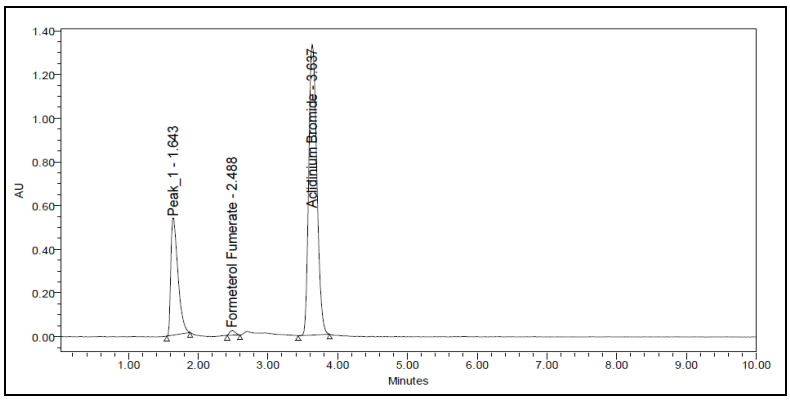


FIG. 10: ACHROMATOGRAM OF ACID HYDROLYSIS (2N HCL)

Base Hydrolysis: Both the drugs were exposed to 2NNaOH, and kept for reflux in Radley apparatus at 70 ^{0}C temperature for 90 min, it showed that 4.95% of Aclidinium bromide and 4.90% of Formoterol Fumarate degradation in base hydrolysis with one degradation products. The obtained chromatogram is shown in **Fig. 11.**

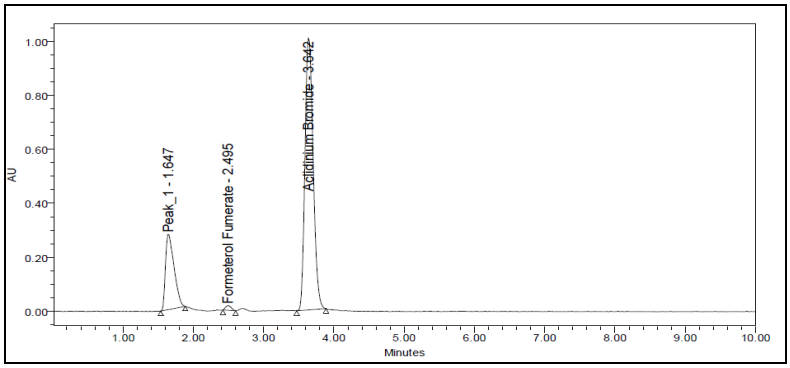


FIG. 11: A CHROMATOGRAM OF BASE HYDROLYSIS (2N NAOH)

Neutral hydrolysis: Both the drugs were exposed to water, kept for reflux in Radley apparatus at 70 °C temperature for 90 min; it was showing that no degradation in neutral hydrolysis. The obtained chromatogram as shown in **Fig. 12.**

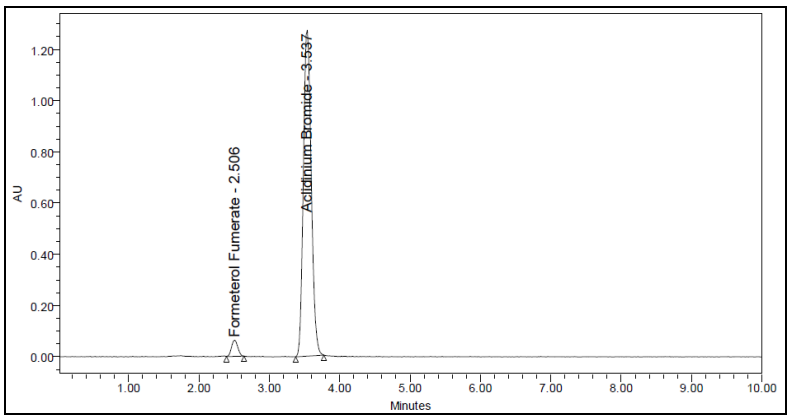


FIG. 12: ACHROMATOGRAM OF NEUTRAL HYDROLYSIS

Oxidative Degradation: Both the drugs were exposed to 3% H_2O_2, at room temperature for 24 hours, 5.96% of Aclidinium bromide and 5.68% of Formoterol Fumarate degradation in 3% H_2O_2 solution at the end of 24 hrs was observed. The obtained chromatogram is shown in **Fig. 13.**

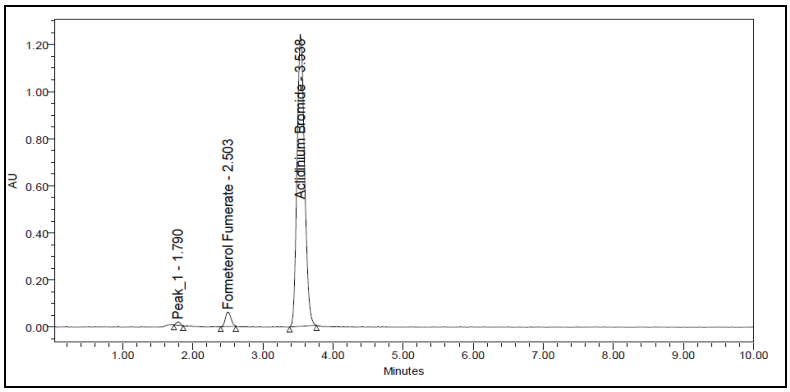


FIG. 13: A CHROMATOGRAM OF OXIDATIVE DEGRADATION (3%H_2O_2)

The blank solutions were also subjected to stress study in the same fashion as the drug solution.

The exposed stress sample and blank solutions were analyzed by HPLC system in all the conditions mentioned above.

Thermal Degradation: Both the drug substances were exposed to 70°C for 24 hrs in a hot air oven; 3.38% of Aclidinium bromide and 2.65% of Formoterol Fumarate degradation was found at the end of 24 hrs of exposure. The obtained chromatogram as shown in **Fig. 14.**

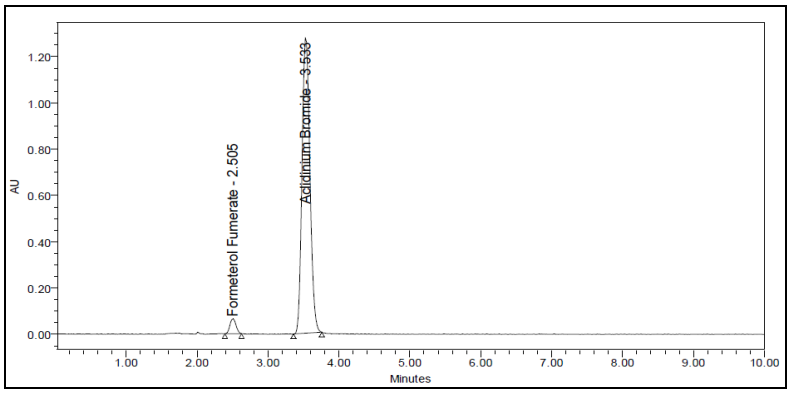


FIG. 14: A CHROMATOGRAM OF THERMAL DEGRADATION

Photo Degradation: Both the drug substances were exposed to direct sunlight for 8 hours; 1.60% of Aclidinium bromide and 1.37% of Formoterol Fumarate degradation was found at the end of 8 hrs of exposure. The obtained chromatogram as shown in **Fig. 15**. The results of degradation studies were mentioned in **Table 8.**

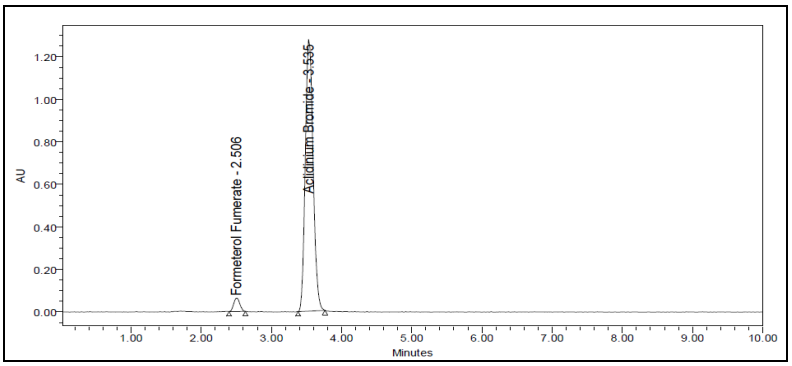


FIG. 15: CHROMATOGRAM OF PHOTOLYTIC DEGRADATION

TABLE 8: SUMMARY OF DEGRADATION STUDIES

S. no.	Condition of degradation study	% of Aclidiniumbromide degraded	% Of Formoterol Fumarate degraded	Retention time of degradant
1.	2N HCl, 90 min	6.34%	6.11%	1.643min
2.	2N NaOH, 90 min	4.95%	4.90%	1.647min
3.	Neutral hydrolysis, 90 min	No degradation	No degradation	-
4.	Oxidative degradation, 24 h	5.96%	5.68%	1.70min
5.	Thermal degradation, 24 h	3.38%	2.65%	-
6.	Photo degradation, 8 h	1.60%	1.37%	-

Method Validation:

Specificity: Retention times of Formoterol and Aclidinium were 2.350 min and 3.412 min, respectively. Did not found any interfering peaks in blank and placebo at retention times of these drugs in this method. So, the proposed method was said to be specific. The obtained chromatograms were as shown in **Fig. 16A, 16B** & **16C.**

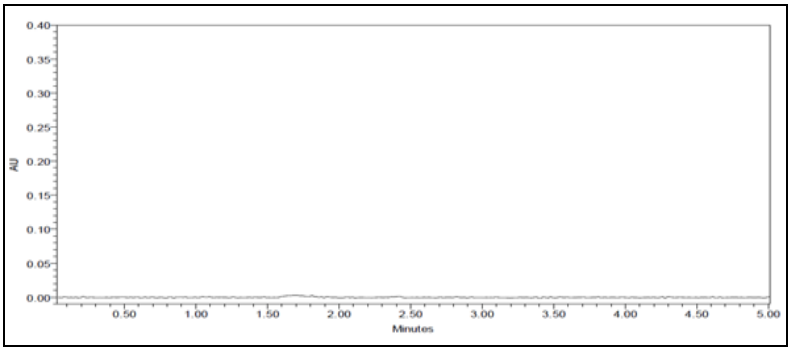


FIG. 16(A): CHROMATOGRAMOF BLANK

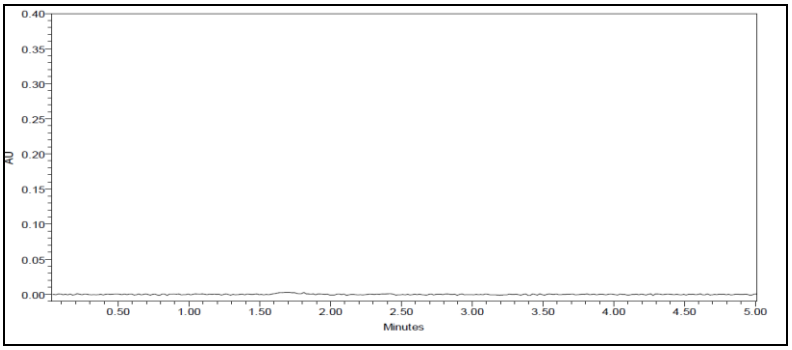


FIG. 16(B): CHROMATOGRAMOF PLACEBO

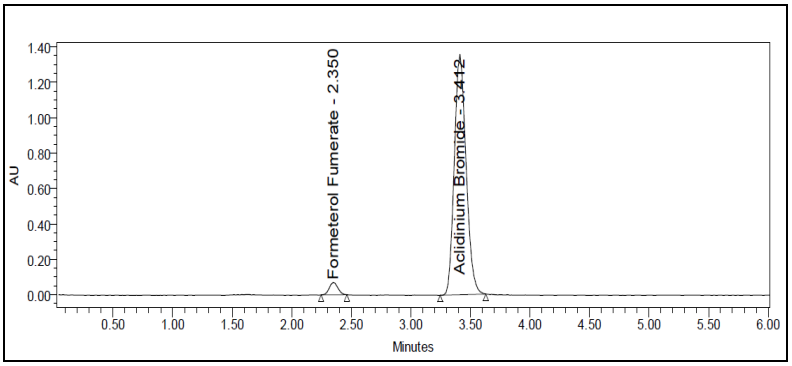


FIG. 16(C): TYPICAL CHROMATOGRAM

Retention times of Formoterol and Aclidinium were 2.350 min and 3.412 min, respectively. Did not found any interfering peaks in blank and placebo at retention times of these drugs in this method. So, the proposed method was said to be specific. The obtained chromatograms were as shown in **Fig. 16A, 16B** & **16C.**

Linearity: Six linear concentrations of Formoterol (1.5-9µg/ml) and Aclidinium (50-300µg/ml) were injected six times. The average areas mentioned above and linearity equations obtained for Formoterol were y =57695x + 4535.1 as shown in Fig. 17 and for Aclidinium y =46398x +50500 as shown in **Fig. 18**.

The correlation coefficient obtained was 0.999 for the two drugs. The obtained results were as shown in **Table 9.**

TABLE 9: LINEARITY TABLE FOR FORMOTEROL AND ACLIDINIUM

Formoterol		Aclidinium	
Conc (µg/mL)	Avg Peak area(n=6)	Conc (µg/mL)	Avg Peak area(n=6)
1.5	86926	50	2389865
3	174305	100	4660978
4.5	271644	150	6960317
6	359391	200	9360350
7.5	431453	250	11759430
9	520886	300	13890150

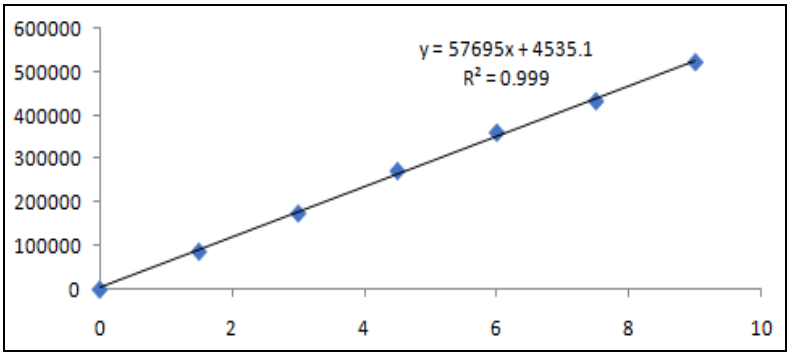


FIG. 17: CALIBRATION CURVE OF FORMOTEROL

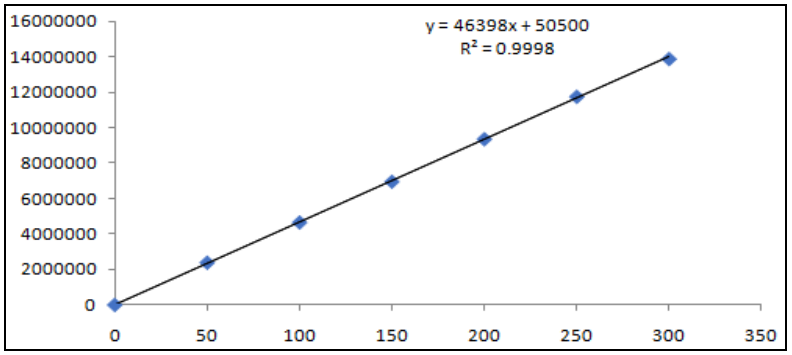


FIG. 18: CALIBRATION CURVE OF ACLIDINIUM

Precision:

System Precision: Six injections were given from a single volumetric flask of working standard solution, and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 1.0% and 0.5%, respectively, for Formoterol and Aclidinium. As the limit of precision was less than "2" the system precision was passed in this method. The obtained results were as shown in **Table 10.**

TABLE 10: SYSTEM PRECISION TABLE OF FORMOTEROL AND ACLIDINIUM

S. no.	Area of Formoterol	Area of Aclidinium
1.	346573	9241736
2.	350884	9342941
3.	351792	9223810
4.	345411	9257503
5.	344686	9307162
6.	342751	9331867
Mean	347016	9284170
S.D	3582.3	49825.2
%RSD	1.0	0.5

Precision: Multiple sampling from a same stock solution was done and six working sample solutions of same concentrations were prepared, each injection from each working sample solution was given on same day and three successive days of the sample preparation and obtained areas were mentioned in the above **Table 11**. Average area, standard deviation and % RSD were calculated for two drugs. As the limit of precision was less than "2" the precision was passed in this method.

TABLE 11: PRECISION TABLE OF FORMOTEROL AND ACLIDINIUM

S. no.	Name of the Drug	Intraday precision			Inter day Precision		
		Mean	SD	%RSD	Mean	SD	%RSD
1	Formoterol fumarate	345136	940.8	0.3	304759	2896.5	1.0
2	Aclidinium bromide	9290851	67438.5	0.7	9193650	35087.6	0.4

Accuracy: Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy, and mean %Recovery was obtained as 100.40% and 99.94% for Formoterol and Aclidinium, respectively. The results obtained were as shown in **Tables 12** & **13.**

TABLE 12: ACCURACY TABLE OF FORMOTEROL

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	3	3.01	100.54	100.40%
	3	3.03	101.30	
	3	2.98	99.37	
100%	6	5.94	99.17	
	6	6.08	101.34	
	6	6.04	100.83	
150%	9	9.05	100.65	
	9	8.94	99.39	
	9	9.09	101.03	

TABLE 13: ACCURACY TABLE OF ACLIDINIUM

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	100	99.60	99.60	99.94%
	100	101.42	101.43	
	100	99.36	99.37	
100%	200	198.26	99.13	
	200	200.66	100.33	
	200	197.92	98.96	
150%	300	299.51	99.84	
	300	301.13	100.38	
	300	301.34	100.45	

Sensitivity:

TABLE 14: SENSITIVITY TABLE OF FORMOTEROL AND ACLIDINIUM

Name of the drug	LOD (µg/ml)	LOQ (µg/ml)
Formoterol	0.03	0.17
Aclidinium	0.99	2.97

Robustness:

TABLE 15: ROBUSTNESS DATA FOR FORMOTEROL AND ACLIDINIUM

S. no.	Condition	%RSD of Formoterol	%RSD of Aclidinium
1	Flow rate 0.8ml/min	0.9	0.6
2	Flow rate 1.2ml/min	0.7	0.3
3	Mobile phase 60B:40A	0.8	0.6
4	Mobile phase 50B:50A	0.6	0.4
5	Temperature 27°C	0.8	0.3
6	Temperature 33°C	1.1	0.6

Robustness conditions like flow rate (±0.2), % of organic content in the mobile phase (±2%) and column temperature (±3°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

Assay: (Duaklir pressair) bearing the label claim Formoterol 12mcg, Aclidinium 400mcg. An assay was performed with the above formulation. % Assay for Formoterol and Aclidinium obtained was 99.91% and 99.99%, respectively. The chromatogram of the formulation is shown in **Fig. 19.**

TABLE 16: ASSAY DATA OF FORMOTEROL

Name of the drug	Label claim (mcg)	Estimated amount (n=6) (mcg)	%Assay
Formoterol fumarate	12	11.99	99.91%
Aclidinium bromide	400	399.97	99.99%

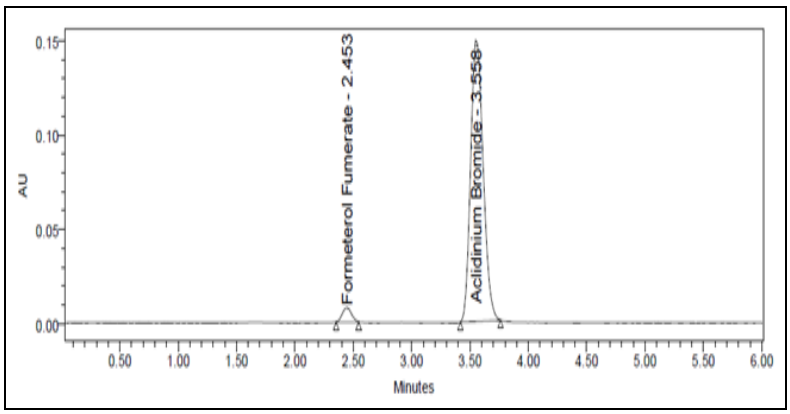


FIG. 19: CHROMATOGRAM OF FORMULATION

CONCLUSION: A simple analytical and robust HPLC method was developed to determine Formoterol Fumarate and Aclidinium bromide using the QbD approach using Design Expert® software, which is capable of separating drug substances from the degradation products. Stress degradation studies have been performed for the drug by using various stress conditions. No degradation products were found in the case of neutral hydrolysis, photodegradation, and thermal degradation. One degradation product was found in acidic, basic, and oxidative conditions. Results obtained from the validation of the developed analytical method were within the limit as per ICH guidelines.

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CONFLICTS OF INTEREST: Nil

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