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Applications of Six Sigma and Quality by Design Approach in UHPLC Method Development for Simultaneous Assessment of Eight Bronchodilators in Drug Substance

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This work portrays the development and validation of a simple and fast UHPLC method for the simultaneous estimation of eight bronchodilators in the drug substance. Process and performance capability (Cpk, Ppk, PPM) studies were conducted by which it may easily conclude how well developed method meeting the requirements. Integration of quality by design (QbD) concept to analytical method development was also discussed. The temperature and mobile phase interaction on chromatographic separation were studied using design of experiments (DoE). Using the QbD and design of experiments (DoE) as combination, a design space was established for developed method. The developed method facilitates the quantitative determination of clenbuterol hydrochloride, fluticasone, formoterol, glycopyrrolate, levalbuterol, metaproterenol, salmeterol and theophylline as a drug substance. All the compounds were separated within 20 min using analytical column X-Bridge BEH C18; (100 × 2.1 mm, 2.5 µ) mobile phase consisted variable mixtures of mobile phase A (10 mM ammonium acetate pH 4.5) and mobile phase B (methanol) with the gradient elution, column temperature 30 ºC, mobile phase flow rate 0.2 mL min/min, wavelength of detection 234 nm.

Keywords: UHPLC, Clenbuterol hydrochloride, Fluticasone, Formoterol, Flycopyrrolate, Levalbuterol, Metaproterenol, Salmeterol.

INTRODUCTION

Bronchodilators are a class of medicine that facilitates respiration, by extending the respiratory systems (bronchi), to relax the lungs muscles. These types of drugs have been used to treat asthma, chronic obstructive pulmonary disease (COPD), allergic reactions, *etc.* Bronchodilator drugs can be defined as drugs with an ant bronchoconstrictor effect. This property can be most easily demonstrated by use of an isolated airway smooth muscle preparation from animal or human [1], these are essential medicines and the action will start very quickly by opening airways. These compounds are available in single dosage forms or in different combinations according to the pulmonary disease requirement. There have been few recent reports [2-7], which provide guidance for simultaneous estimation using traditional chromatographic methods such as HPLC, GC and other spectroscopic techniques, either alone or in combination with other techniques.

Analytical method development is an integral part of the manufacturing process to determine the identity, purity and potency of the compound that was prepared. Therefore, it is important to develop a robust method which delivers the intended purpose consistently. There are many factors, including basic parameters (*e.g.* physico-chemical properties, material nature, solution stability), internal parameters (*e.g.* method parameter) and external parameters (*e.g.* environmental factors, instrument models, reagent quality and analytical skills) that should be considered while developing a robust analytical method [8].

In view of all these obstacles, the quality by design (QbD) approach has been used for the current analysis. Pharmacopeia and several regulatory authorities provide regulatory guidance and stress the importance of QbD in the production of pharmaceuticals. Using the enhanced QbD approach, we can easily define critical operating parameters that are useful for method development, execute multivariate experiments to understand the product and process and build the design space [9]. Design space helps to define the critical parameter that affects the developed method. The objective of each chromatographer is to develop a robust method that is intended to serve the purpose.

Today, too many process failures occurred at the time of method transfer or at the quality control facility which are frequently leading to expensive repetitions, such as redevelopment and revalidation. To avoid this robustness testing becomes an integral part of method development and this study helps us to create space for design [10-16].

A comprehensive literature survey reveals that few techniques have been documented for determination and quantification of bronchodilator using liquid chromatography with mass spectroscopy, ultraviolet absorption spectrometry, thin layer chromatography either alone or in combination with other drug material [2-7]. Thus, in this work, a single UHPLC method is developed that can be used for the simultaneous estimation of few bronchodilators *e.g*. clenbuterol hydrochloride, fluticasone, formoterol, glycopyrrolate, levalbuterol, metaproterenol, salmeterol and theophylline (Fig. 1).

Once after method development, we should ensure that it is suitable for the intended purpose. To monitor this, process capability indices are used to check the effectiveness of developed method. The process capacity indices, like Cp and Cpk, were commonly used in tools that will provide numeric measurements, whether the process could reproduce products within the defined specification limits. The Cp values take account of process variability compared to output tolerance, which represents the consistency of product quality and future process capabilities. The Cpk measures the extent of process change and process deviation from the actual value [17-19]. This simulated data is used for the calculation of capability analysis also process performance index is a statistical tool used to verify that the sample manufactured by the process will meet the specifications. In simple terms, Ppk is a process efficiency index that indicates how well the system meets the requirements and how well the procedure focuses on the requirements.

EXPERIMENTAL

Chromatographic grade reagents *viz*. methanol, ammonia acetate, acetic acid, manufactured by Merck, were used for preparation of mobile phase, samples and standard solutions.

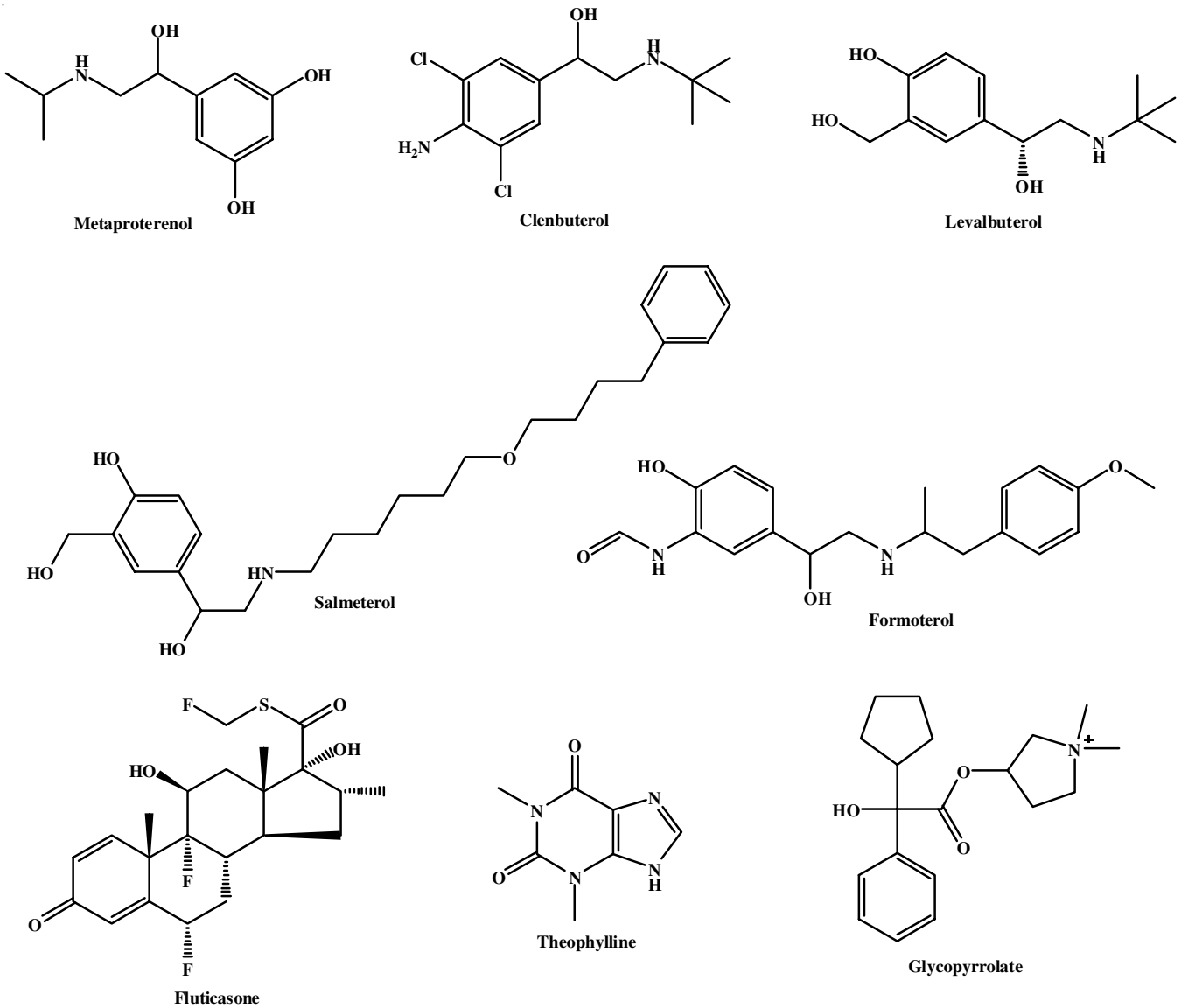

Fig. 1. Structure of bronchodilators

Method: Waters H-Class UPLC system (Acquity UPLC QSM, Waters/186015018) comprise a quaternary solvent, sample manager with flow through needle, column manager and PDA detector which are controlled by empower software was used for all chromatographic separations. Various types of columns with chemistry C18, C8 amino were used for method development and finally X-Bridge BEH C18; 100 × 2.1 mm, 2.5 µ, column was used for development and validations. Design expert version- 8.07.1 (Stat-Ease, Inc. Minneapolis, USA) software used to study the effect of chromatographic parameters like buffer strength, column temperature and flow rate on developed method.

Chromatographic conditions: UPLC system equipped with (PDA with 190-400 nm @235 nm) PDA detector, X-Bridge BEH C18; 100 × 2.1 mm, 2.5 µ column used for the chromatographic separations. The flow rate and column temperature were about 0.2 mL/min and 30 ºC, respectively. Variable mixtures of mobile phase A (10 mM ammonium acetate pH 4.5) and mobile phase B (methanol) with the gradient elution as follows 0 to 2 min, 75% A; 2 to 5 min, 75% to 60% A; 5 to 9 min, 60% A; 9 to13 min, 20 to 20% A; 13 to 17 min, 20% A; 17 to 17.1 min, 20 to 75% A and then 17.1 to 20 min, 75% A.

Mobile phase preparations

Mobile phase A: 10 mM Ammonium acetate pH 4.5 prepared by dissolving about 0.77 g of ammonium sulfate in 1000 mL water and adjusted to pH 4.5 with acetic acid.

Mobile phase B: 100% Methanol.

Diluent: Prepared a mixture of acetonitrile and water in the ratio 50:50.

System suitability: Suitable amount of clenbuterol hydrochloride (CLN), fluticasone (FLU), formoterol (FMT), glycopyrrolate (GPL), levalbuterol (LBL), metaproterenol (MPN), salmeterol (SLM) and theophylline (TPY) were weighed and dissolved in diluent to get a solution having concentration about 50 µg/mL.

Assay preparations: For assay, suitable amount of clenbuterol hydrochloride (CLN), fluticasone (FLU), formoterol (FMT), glycopyrrolate (GPL), levalbuterol (LBL), metaproterenol (MPN), salmeterol (SLM) and theophylline (TPY) were weighed and dissolved in diluent to get a solution having concentration about 50 µg/mL.

Method validations: The developed method was validated for system suitability, linearity, accuracy and robustness.

Robustness studies: The robustness of the method developed was investigated using full factorial studies. The interaction effect of the variable parameters was studied through deliberate parameter changes in experimental conditions and system suitability parameters such as resolution, retention time tailing and others were measured. Flowrate, temperature and gradient composition have significantly impacted on the separations based on preliminary studies during method development, hence these three experimental parameters were considered for robustness study. Using design expert a multi-dimensional design space was constructed to study the robustness of the method developed. A 2 level factorial design used to build the mathematical models (Table-1). resulting 2^3 (18) experiments were conducted as per designed model and in all experiments the resolution was > 2 between the impurities and tailing factor was < 1.3. To study the effect of flow rate on developed method flow rate was changed to 0.18 mL/min and 2.2 mL/min in instead of 0.2 mL/min (flow rate change to ± 10%), to verify the effect of column temperature and pH method was studied at a temperature between 35 to 25 ºC and pH 4.3 to 4.7.

RESULTS AND DISCUSSION

Method development: The aim of this study is to develop a robust UHPLC method for the simultaneous estimation of clenbuterol hydrochloride (CLN), fluticasone (FLU), formoterol (FMT), glycopyrrolate (GPL), levalbuterol (LBL), metaproterenol (MPN), salmeterol (SLM) and theophylline (TPY). Method development initiated using advanced chemistry development (ACD) software, using this software were extracted all physico-chemical properties such as pK_a (acid), pK_a (base), log P, log D, molecular weights, hydrogen bonding donor and acceptor power, based on this information a suitable working range such as pH and columns was identified. Acceptable pH intervals were 1.9-2.4, 4.8-5.3, 7.3-7.9, 9.6-10.1, 10.2-10.6 and columns like C18, C8, amino and phenyl can be used. It was very challenging to identify the choice column due availability of huge no of columns from different brands, the choice of buffer and solvent was limited. To start with the initial screening or method optimization we used different combination of buffers (0.1% formic acid and ammonium acetate pH4.5) and solvents (acetonitrile and methanol) and columns having different stationary phase like C18, C8 and phenyl were used for initial screening. Using pilot studies data, we recognized that there was poor separation or low resolution in C8 and phenyl columns and substantial separation was observed in C18 columns. The peak tailing and splitting with 0.1% formic acid and acetonitrile in the subsequent optimization studies were also observed. Considering the aforementioned data C18 (X-Bridge BEH C18; 100 × 2.1 mm, 2.5 µ) column and 10 mM ammonium acetate; pH 4.5 buffer in combination with methanol was used to further optimize the method using gradient elution. Using 10 mM ammonium acetate, pH 4.5 buffer in combination with methanol was used to fine-tune or finalize the opti-

TABLE-1
DESIGN SUMMARY
File version: 8.0.7.1, Study type: Factorial, Design type: 2 Level Factorial, Center points: 2,
Design model: 3FI, Runs: 18, Blocks: No blocks, Build time (ms): 1.95594

Factor	Name	Units	Type	Subtype	Minimum	Maximum	Mean	Std. Dev.
A	Flow rate	mL/min	Numeric	Continuous	0.18	0.22	0.2	0.02
B	Temperature	%	Numeric	Continuous	25	35	30	7.07
C	pH	NA	Numeric	Continuous	4.3	4.7	4.5	0.28

mized method with a different combination of gradients and all findings from these tests are given in Table-2 and finalized method conditions as mentioned in chromatographic conditions.

Method validation

System suitability: The results of the system suitability were measured from six standard solution replicates (Fig. 2),

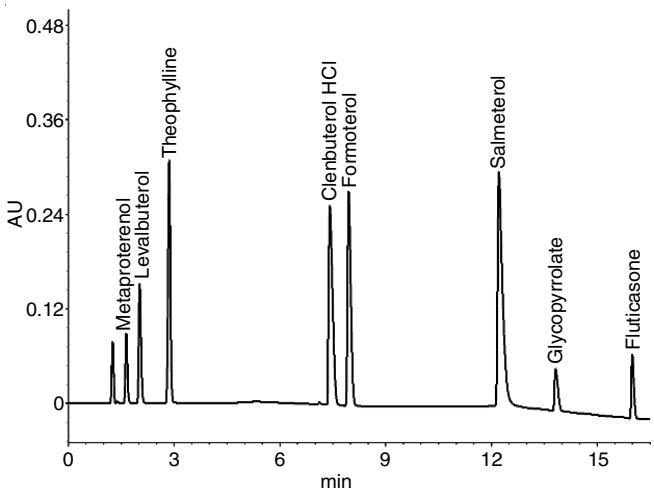

Fig. 2. Standard solution chromatograms from final conditions

resolution between adjacent bronchodilator peaks, tailing factor, theoretical plates, % of RSD were calculated and the results obtained are summarized in Table-3.

Specificity: Method specificity was proved from the results of peak purity obtained from chromatogram obtained using PDA detector, where all 8 peaks were resolved within 20 min. From the UV spectra of all eight components obtained purity threshold was found higher than angle which indicates that the peak is pure. The obtained results are summarized in Table-4.

Precision and intermediate precision: The %RSD was calculated using six replicate injection of standard solution and the values were >2.0% for CLN, FLU, FMT, GPL, LBL, MPN, SLM and TPY. The %RSD obtained from the intermediate precision studies was about 0.5% for the peak areas of CLN, FLU, FMT, GPL, LBL, MPN, SLM, TPY, which confirmed the precision of developed analytical method. The obtained results are summarized in Table-4.

Accuracy: The %recovery of CLN, FLU, FMT, GPL, LBL, MPN, SLM, TPY is between 98.59 to 101.64, which is well within the acceptance criteria. These low percent RSD obtained proved the accuracy of the developed method (Table-4).

Linearity: Linearity studies of the developed method was established using standard solution with a concentration ranging from 25 to 150% of standard solution concentration, which is

TABLE-2
METHOD OPTIMIZATION RESULT

Column	Mobile phase	Elution mode	Observation	Result
C18	Ammonium acetate pH4.5 and acetonitrile (50:50 v/v)	Gradient: 50:50% for 2 min 50-90%B in 10 min 10:90% for 15 min	LBL and TPY, SLM and GPL were co-eluted	Rejected
C18	0.1% Formic acid and acetonitrile (90:10 v/v)	Gradient: 90:10% for 2 min 10-90%B in 10 min 10:90% for 15 min	MPN and LBL not resolved (co-eluted) CLN and FMT not resolved (co-eluted) SLM, GPL, FLU not resolved (co-eluted)	Rejected
C8	Ammonium acetate pH4.5 and acetonitrile (50:50 v/v)	Gradient: 70:30% for 2 min 30-70% B in 10 min 30:70% for 6 min	LBL and TPY not resolved (co-eluted) CLN and FMT not resolved (co-eluted) SLM and GPL not resolved (co-eluted)	Rejected
C18	Ammonium acetate pH4.5 and methanol (70:30 v/v)	Gradient: 70:30% for 2 min 40-60%B in 4 min 40:60% for 8 min	MPN and LBL not resolved (co-eluted) CLN and FMT resolved (valley points observed)	Rejected
C18	Ammonium acetate pH4.5 and acetonitrile (80:20 v/v)	Gradient: 80:20% for 2 min 3%B in 4 min 40:60% for 8 min	SLM, GPL, FLU does not resolve (co-eluted)	Rejected
C18	Ammonium acetate pH4.5 and methanol (80:20 v/v)	Gradient: 80:20% for 1 min 40-60%B in 6 min 40:60% for 9 min	Observed peaks were sharp and almost all peaks were resolved	Rejected
C18	Ammonium acetate pH4.5 and acetonitrile (70:30 v/v)	Gradient: 70:30% for 2 min 40-60%B in 4 min 40:60% for 8 min	MPN and LBL not resolved(co-eluted) CLN and FMT resolved valley points observed)	Rejected

TABLE-3
SYSTEM SUITABILITY RESULTS FROM STANDARD SOLUTION

Name	Retention time	USP resolution	USP tailing	USP plate count	%RSD
Metaproterenol	1.643		1.22	4284	0.46
Levalbuterol	2.021	3.37	1.34	4502	0.42
Theophylline	2.856	6.84	1.12	9088	0.21
Clenbuterol HCl	7.417	26.91	1.85	18875	0.19
Formoterol	7.95	2.68	1.56	31950	0.37
Salmeterol	12.211	18.89	2.37	34889	0.29
Glycopyrrolate	13.82	7.05	1.69	90096	0.46
Fluticasone	15.993	13.5	1.45	223229	0.42

TABLE-4
SUMMARY OF VALIDATION RESULTS

Parameter	MPN	LBL	TPY	CLN	FMT	SLM	GPL	FLU
Linearity and range (%)	25 to 150%	25 to 150%	25 to 150%	25 to 150%	25 to 150%	25 to 150%	25 to 150%	25 to 150%
Slope	6789174.857	13826208.57	29649321.71	39004815.43	35156396	59406429.14	6983645.714	8369629.714
Intercept	-1350.4	2800	-32049.86667	4069.866667	16047.46667	-21537.06667	701	-4599.8
Correlation-coefficient	1.0000	0.9999	0.9993	0.9997	0.9998	0.9999	0.9999	0.9998
Accuracy at 50 (%) level								
% Recovery	99.45	99.25	100.03	99.36	99.62	99.23	99.89	98.99
% RSD	0.45	0.27	0.23	0.28	0.33	0.45	0.23	0.36
Accuracy at 100 (%) level								
% Recovery	99.18	99.23	99.86	99.45	99.73	99.65	99.79	99.92
% RSD	0.27	0.66	0.36	0.65	0.38	0.49	0.25	0.22
Accuracy at 150 (%) level								
% Recovery	99.89	99.35	99.87	99.66	99.58	99.45	99.79	99.73
% RSD	0.39	0.35	0.24	0.41	0.18	0.46	0.23	0.37
Intermediate precision								
% Assay	99.86	99.79	99.37	99.56	99.78	99.12	99.89	99.45
%RSD	0.44	0.52	0.28	0.36	0.19	0.46	0.31	0.19
Specificity								
Purity1 threshold	1.095	0.851	0.691	0.736	0.682	0.766	2.309	1.466
Purity1 angle	0.751	0.058	0.034	0.044	0.041	0.168	0.068	0.066

from 0.0125 to 0.075 mg/mL. The correlation coefficient values were obtained from the linear calibration plot over a calibration range of 25% to 150%, which was found to be greater than 0.999 (Table-4).

Design of experiments for robustness study: A mathematical model was developed using design expert software to conduct robustness study for the method developed. Full factorial experimental design with 18 experiments (2 levels and 3 factors) performed considering flow rate (mL/min), temperature (ºC) and pH as critical parameters (Table-2). Criteria of resolution (< 2.0) were considered as response to assess the impact of the factors and performed experiments in the asymmetrical order to diminish the errors from uncontrolled factors that may influence the responses. The influence of these variables was measured and the results were calculated. The resolution and tailing factor obtained should be no less than 2.0 and not more than 1.5, respectively. The lowest resolution found was 2.1 and all the experiments had a tailing factor of less than 1.5. The developed method will therefore be considered as robust, as the failure rate in the design space studied was 0%.

To study the significance of the model, statistical methods known as Analysis of Variance (ANOVA) were used, all results of ANOVA are reported in Table-5. The obtained "P value > F" and p value less than 0.0500 indicate model was significant. Creating the perturbation plots (Fig. 3) it's been concluded that all three parameters will have a significant impact on the resolutions except the resolution between FMT and SLM, SLM and GPL. After analyzing the Cube plots designed to represent the effects of three variable at a time (Fig. 4), the effect of all factor was indicated to be largely independent of resolution.

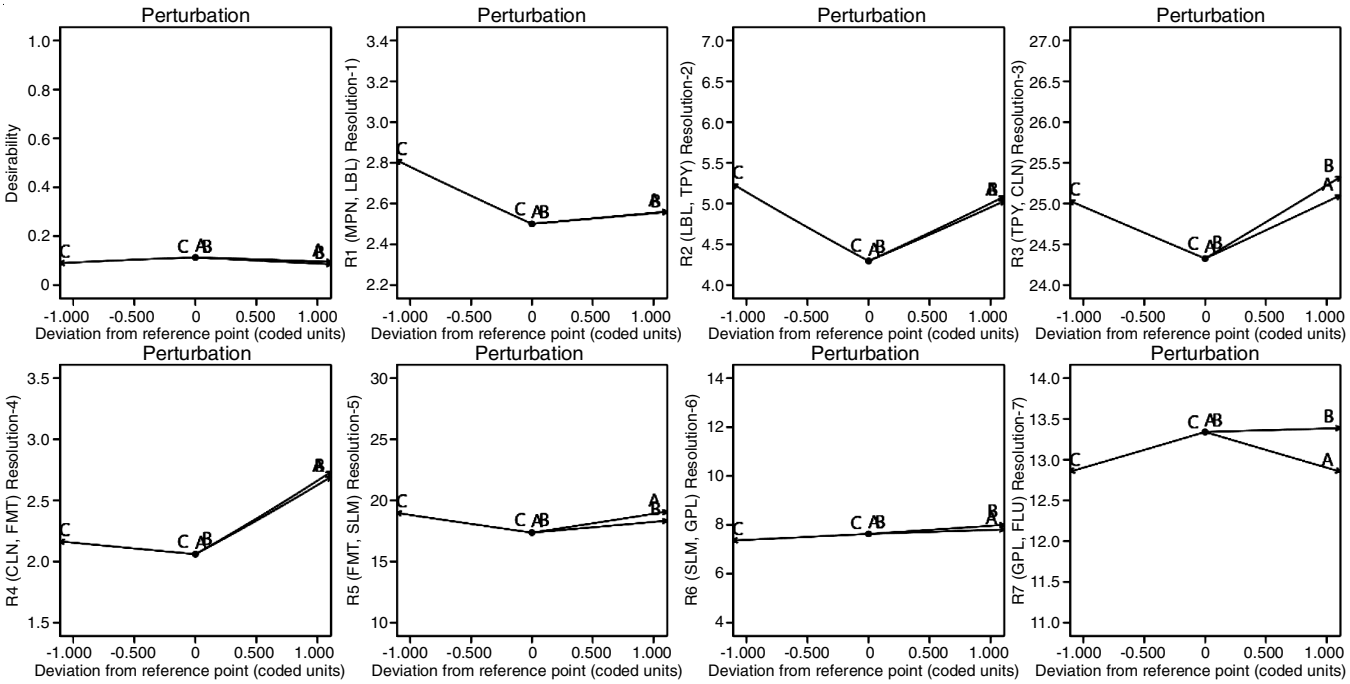

Fig. 3. Perturbation plot for the resolution as reponse (factor: flow rate, temperature, pH)

TABLE-5
ANOVA RESULTS FOR RESPONSE (RESOLUTIONS) OBTAINED FROM EXPERIMENTAL DESIGN

	Source	Sum of squares	Df	Mean square	Model F-value	Model p-value	Prob > F
Resolution-1 (MPN, LBL)	Model	0.0857	5	0.0167	40.49	< 0.0001	Significant
	Flow rate	0.0072	1	0.0072	17.48	0.0019	
	Temperature	0.0152	1	0.0152	36.87	0.0001	
	pH	0.0505	1	0.0505	122.48	< 0.0001	
Resolution-2 (LBL, TPY)	Model	0.2727	7	0.0390	258.63	< 0.0001	Significant
	Flow rate	0.0084	1	0.0084	55.43	< 0.0001	
	Temperature	0.0056	1	0.0056	37.04	0.0003	
	pH	0.0362	1	0.0362	240.33	< 0.0001	
Resolution-3 (TPY, CLN)	Model	0.0594	6	0.0099	14.30	0.0004	Significant
	Flow rate	0.0015	1	0.0015	2.10	0.1808	
	Temperature	0.0066	1	0.0066	9.46	0.0132	
	pH	0.0092	1	0.0092	13.32	0.0053	
Resolution-4 (CLN, FMT)	Model	0.3979	5	0.0796	116.55	< 0.0001	Significant
	Flow rate	0.0037	1	0.0037	5.39	0.0427	
	Temperature	0.0001	1	0.0001	0.1961	0.6673	
	pH	0.0088	1	0.0088	12.84	0.0050	
Resolution-5 (FMT, SLM)	Model	3.52	6	0.5861	1465.54	< 0.0001	Significant
	Flow rate	0.0097	1	0.0097	24.15	0.0008	
	Temperature	0.0087	1	0.0087	21.75	0.0012	
	pH	2.07	1	2.07	5171.66	< 0.0001	
Resolution-6 (SLM, GPL)	Model	2.83	6	0.4709	109.44	< 0.0001	Significant
	Flow rate	0.0616	1	0.0616	14.33	0.0043	
	Temperature	0.1211	1	0.1211	28.16	0.0005	
	pH	1.64	1	1.64	381.06	< 0.0001	
Resolution-7 (GPL, FLU)	Model	0.1303	5	0.0261	44.39	< 0.0001	Significant
	Flow rate	0.0233	1	0.0233	39.76	< 0.0001	
	Temperature	0.0223	1	0.0223	37.90	0.0001	
	pH	0.0717	1	0.0717	122.02	< 0.0001	

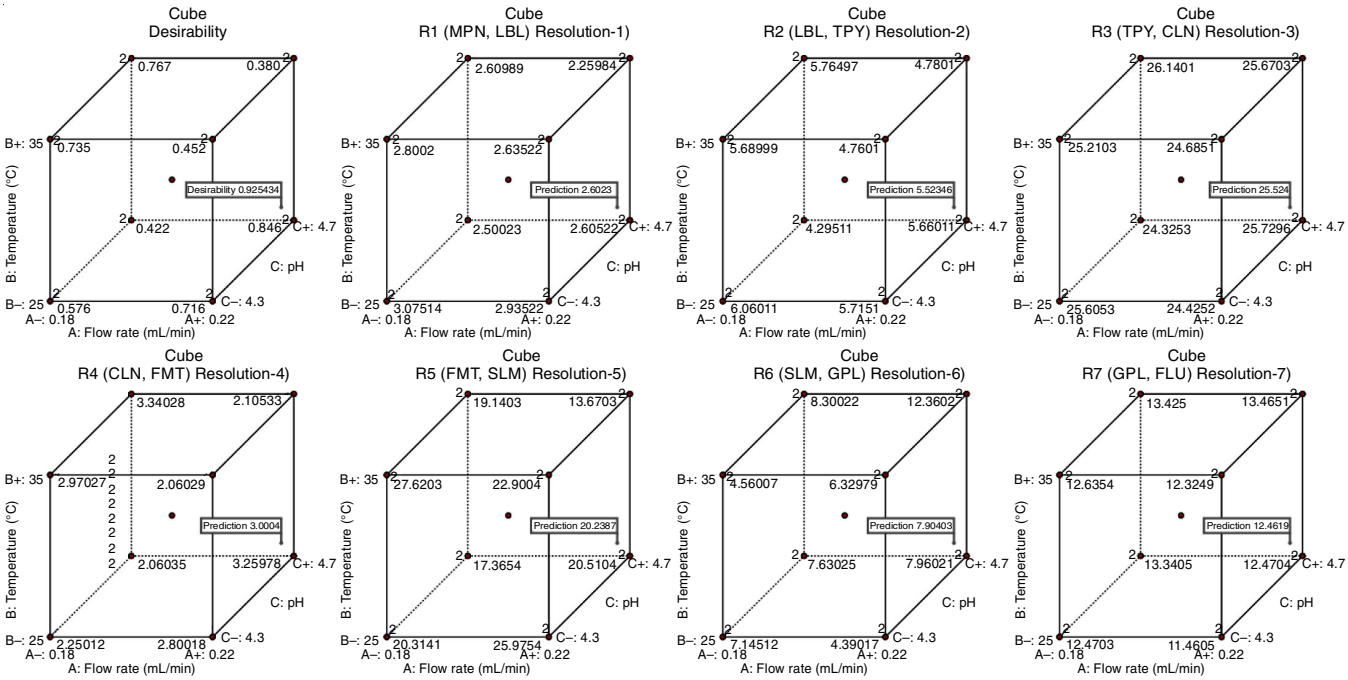

Fig. 4. Cube plots designed for the resolution as reponse (factor: flow rate, temperature, pH)

and all of the (resolution) response was above the predicted value. Hence, the developed method can considered as robust.

The contour plots were used to understand better the data, which provided information on critical parameters such as resolution considering flow rate, temperature and pH as critical method attributes. By studying the contour plot, it was clear that when the working region moves from blue to red, resolution will increase. Fig. 5 represents the contour plot for all the resolutions considered flowrate and temperature as critical method parameters. These plots were also used for calculating

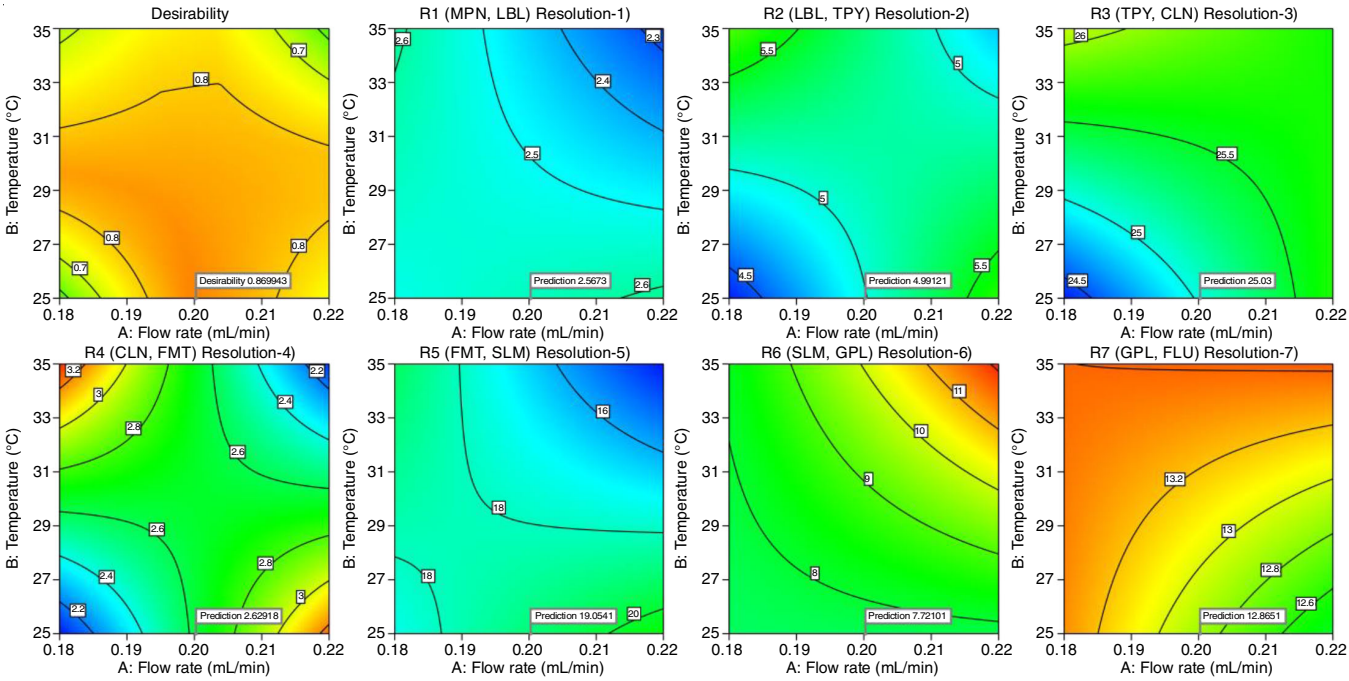

Fig. 5. Contour plot for resolutions considering flowrate and temperature as critical method parameters

disability. The contour plots indicated that robustness studies obtained the maximum desirability.

Performance capability studies: Fig. 6 indicates the probability plots for the measurement of distribution normality. The p-values of all experiments were larger than 0.5, indicating that the data were normally distributed at 5%. Therefore, normal distribution capacity analysis has been carried out and for the experiments where the P value is less than 0.5, the values were adjusted with Boc-Cox transformation to calculate the distribution. Table-5 displayed the results of the various capacity analysis and process performance indices from all experiments. All the indices should be higher than 1.33, if the method is to generate within specification. Table-6 shows that all the indices were above 1.33, which shows that the process passes the capacity analysis and the developed method was able to generate the results which meet the requirements. Moreover, the Cpk and PPM both measures the defects in the method, PPM stands for the defective parts of the method. The higher the index, the closer the process operates and the lower the defective parts per million. The higher Cpk, Ppk and lower PPM values indicated that the developed method was fit for the intended purpose. The Cpk and Ppk values are quite similar, indicating that the operation is under static control. Overall the developed method is suitable for the intended purpose.

Conclusion

A UHPLC method for the simultaneous estimation of bronchodilators (*e.g.* clenbuterol hydrochloride, fluticasone, formoterol, glycopyrrolate, levalbuterol, metaproterenol, salmeterol and theophylline) was successfully developed and validated as per the guidelines. The experimental designs used to verify robustness of developed method. Based on the summary of validation results, the method was accurate and robust. Moreover, the results from process and performance capabilities studies confirms the method is suitable for its intended purpose.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

TABLE-6
PROCESS AND PERFORMANCE VALUES AS A FUNCTION OF THE SPECIFICATION LIMITS FOR A KNOWN PROCESS PERFORMANCE

Source	Std. Dev.	Short term statistics			Long term statistics		
		Cpk	ppm	Method capability	Ppk	ppm	% Output within specification
Resolution-1 (MPN, LBL)	0.01010	2.16	0.00	Excellent	2.79	0.00	99.99%
Resolution-2 (LBL, TPY)	0.68930	1.64	0.46	Satisfactory	1.60	0.84	99.99%
Resolution-3 (TPY, CLN)	0.66990	11.60	0.00	Excellent	10.50	0.00	99.99%
Resolution-4 (CLN, FMT)	0.03255	1.58	1.03	Satisfactory	1.64	0.41	99.99%
Resolution-5 (FMT, SLM)	0.53350	1.94	55.27	Satisfactory	2.35	1.23	99.99%
Resolution-6 (SLM, GPL)	0.31930	1.33	33.72	Capable	1.44	7.43	99.99%
Resolution-7 (GPL, FLU)	0.71780	4.98	0.00	Excellent	5.61	0.00	99.99%

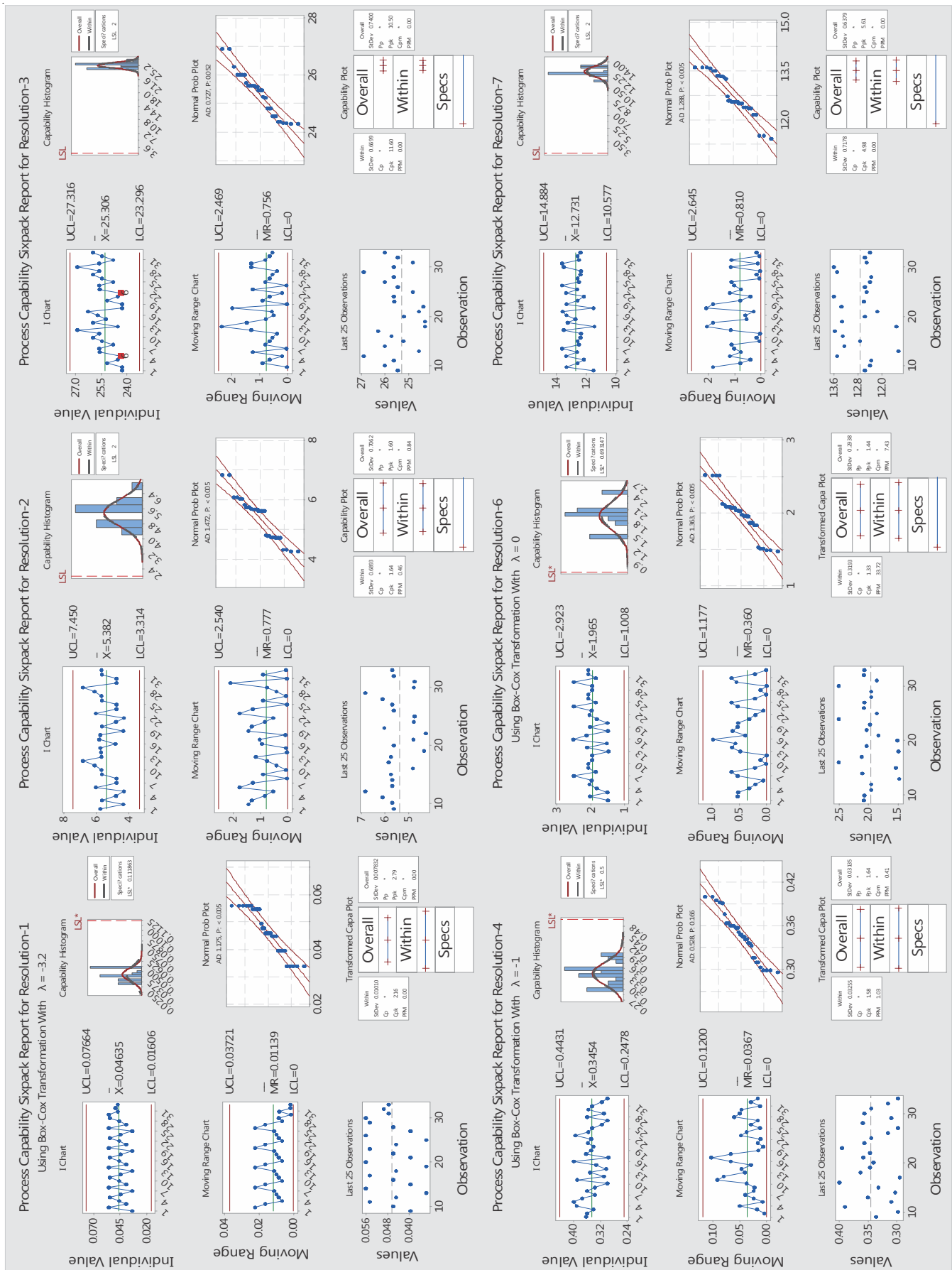

Fig. 6. Indicates the probability plots for the measurement of distribution normality and process and performance capability

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