Synchronized Quantitative Assessment of Corticosteroid and Bronchodilator in Rotacaps by HPTLC using Fractional Factorial Design

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ABSTRACT

Background: A validated high performance planar chromatographic analysis for a combination medication of corticosteroids mometasone furoate and bronchodilators formoterol fumarate available as Evocort®: inhaler in market was developed. It is used for treating asthma, a reversible obstructive airways disease. **Materials and Methods:** The HPTLC chromatographic condition was optimised using aluminium sheets formerly coated with silica gel $60F_{254}$ as stationary phase and toluene: methyl alcohol: methanoic acid (12.5:4:0.3 v/v/v) as mobile phase. **Results:** Formoterol fumarate and mometasone furoate concentration was found to be directly proportional to peak area in the range of 120-720 ng/band and 3996-23976 ng/band, respectively. Results of precision studies were between 0.77-1.274 stated in %RSD and recovery studies ranged from 99-101% indicating very low inter day variability and good reproducibility of the method. Fractional factor design was applied to five factors i.e. volume of mobile phase, amount of methyl alcohol in mobile phase, chamber saturation time, development distance, time from chromatography to scanning to study its effect on retardation factor and peak area of both the drug. **Conclusion:** The 3D response surface graphs exposed that ratio of methyl alcohol in the mobile phase was slightly rigorous factors affecting the responses. The compatibility of linearity ranges with ratio in their combined rotacaps and also nonappearance of excipients interference recommends application of the proposed methods in quality control analysis of cited drugs in commercial rotacaps.

Key words: Analytical method development, Validation, HPTLC, Mometasone furoate, Formoterol fumarate, DoE.

INTRODUCTION

Disease of chronic obstructive airway is predicated to become the third cause of mortality by 2030 according to the World Health Organization.[1] The inflammation in COPD and asthma can be controlled in more efficient manner by straight way delivering the drug in the airways and lungs which will minimize the dose and its side effects.[2] Such drugs can be delivered through pulmonary route for its significant effect. Nebulizers, pressurized metered-dose inhaler (pMDI) and dry powder inhalers (DPIs) are widely used device for the distribution of active drug moiety as aerosols. To deliver powder medication, DPIs are commonly used as it required least patient synchronization between breathing and actuation.[3] Though DPI provide good stability to drug formulation than liquid formulation but it is associated with complication of manufacturing powders with the appropriate characteristics to give ease of aerosolization and alveolar delivery. Evocort® inhaler is a combination medication of corticosteroids mometasone furoate and bronchodilators formoterol fumarate for treating asthma, a reversible obstructive airways disease. These DPIs are formulated as low-drug dose products
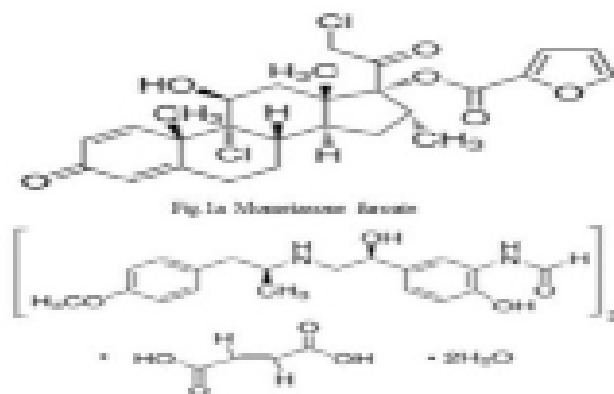
Figure 1: Chemical Structure of (a) Mometasone furoate and (b) Formoterol fumarate.

with microgram doses and it produces the additive effect for improving the symptoms, lung functions and reduces exacerbation in patient.[4,5]

Mometasone furoate (MMF) chemically is 9α,21-dichloro-11β-hydroxy-16α methylpregna-1,4-diene-3,20-dione 17-(2-furoate) (Figure 1). Solubility of MMF is less in alcohol, unsolvable in water and freely dissolved in acetone and dichloromethane. Formoterol fumarate (FRF) chemically is (R*, R*)-N-[2-Hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl) -1-methylethyl] amino] ethyl] phenyl] formamide fumarate, dihydrate (Figure 1). Its show very low ability to dissolved in water and iso propanol; practically insoluble in acetonitrile; soluble in methyl alcohol.[6-10]

Extensive literature survey discloses that estimation of mometasone furoate single or in admixture with other drugs using chromatographic method (HPLC, HPTLC, GC and supercritical fluid) and UV spectrophotometry.[10-21] The estimation of FRF alone and in combination (mixture) with other active drug moiety including HPLC, GC and UV spectrophotometry has stated in published paper.[22-36]

The advancement of planar chromatography i.e., HPTLC has been arose as a chief tool in drug investigation and simplest of all the chromatographic techniques. HPTLC, a separation technique is flexible and rapid for quantitatively evaluation of extensive range of samples. The experimental design approach is the technique of simultaneous examination of the influence of different factors on robustness of the method using a certain plan (matrix) of experiments.[37,38] So, there was a need to develop the new alternative HPTLC method which is more sensitive than, the reported method[39] and the robustness parameters are evaluated with the help of DOE approach.

MATERIALS AND METHODS

Materials

Mometasone furoate (MMF**)** pure drug and Formoterol fumarate (FRF) was supplied as gratis sample from Sun Pharmaceutical Industries Ltd. Evocort® manufactured by Cipla Ltd. was purchased from local market. Toluene, methyl alcohol, ethyl alcohol, N, N-Diethylethanamine, orthophosphoric acid, triethylamine, ethanoic acid, methanoic acid of AR grade was procured from Loba Chemie Pvt. Ltd. Mumbai, India.

Instrument

The HPTLC system (Camag, Mutenz, Switzerland) consisting semi-automatic spotting device, Linomat V, an HPTLC syringe (100 µl capacity) a glass twin-trough TLC chamber (20 × 10 cm) for the development of the TLC plate and a TLC scanner- Camag 3 with winCATS (V 1.4.7) software for the interpretation and evaluation, was used for thin layer chromatographic studies. UV cabinet: 254 nm and 366 nm for detection of spots and Pre-coated TLC plate: Silica gel 60 F_{254} Aluminium backed layer (200 µm) as stationary phase was used in the study.

Procedure for references stock and sample solution

Reference stock solution containing 1000 µg/ml of FRF and 6660 µg/ml of MMF concentration was prepared by weighing 10 mg and 66.6 mg of reference FRF and MMF, in separate 10 ml standard flask using solvent as methyl alcohol. Working standard of concentration 40 and 1332 µg/ml of FRF and MMF was prepared by mixing 1 ml of FRF and 5 ml of MMF stock solution in 25 ml standard flask.

Sample solution

The powder content of forty rotacaps were emptied and accurately weighed. Appropriate quantity of the fine powder (807.6 mg) corresponding to 0.2 mg of FRF and 6.66 mg of MMF was placed to a 10 ml standard flask holding 5 ml of methyl alcohol. The solution was sonicated and methyl alcohol was added up to 10 ml. Contents were filtered through 0.45 µm membrane filter and the conc. of 20 and 666 µg/ml of FRF and MMF, respectively was obtained.

Marketed formulation quantitative analysis

FRF (20 µg/ml) and MMF (666 µg/ml) was extracted from Evocort®. Twelve microliters of the above sample solution were applied on the TLC plate and examined by developed technique. Based on analyte's peak area,

percentage assay of FRF and MMF in the formulation was calculated.

Chromatographic Condition

The HPTLC chromatographic condition was optimised using aluminium sheets previously coated with silica gel 60F$_{254}$ and toluene: methyl alcohol: methanoic acid (12.5:4:0.3 v/v/v) as mobile phase. The mobile phase was allowed to saturate the chamber for 20 min. The band width of 6 mm spotted plates was kept in it and mobile phase was allowed to travel up to 80 mm and then dried for 5 min in oven at 110°C. Thereafter, 220 nm of scanning wavelength was chosen, deuterium lamp was used as radiation source with 40 mm/s of scanning speed and 6 × 0.45 mm dimension of silt. Developed plate was placed in a scanner which help in the estimation of analyte quantitatively by measuring the intensity of diffused reflected light corresponding to peak area and R$_f$ values.

Validation of Chromatographic Method[40]

Specificity of the method was assessed by relating the peak purity of chromatographic peaks and analyzing the R$_f$ value of analyte in the pharmaceutical dosage form with the standard drug solution. The band of FRF and MMF in Evocort® were compared with R$_f$ values and densito-spectra of band of reference drugs.

Linearity between the quantity of analyte and their peak area was assessed by applying different volumes, i.e., 3, 6, 9, 12, 15 and 18 μl (FRF: 120-720 ng/band; MMF: 3996-23976 ng/band) of reference solution (FRF: 40 μg/ml and MMF:1332 μg/ml). The developed plate was analysed and chromatograms were computed. Calibration curve using peak area vs ng/band were sketch for linear representation and slope, intercept and coefficient of determination values were calculated by least square method. The limit of detection and limit of quantification of FRF and MMF were calculated using the equation as mention in ICH guideline i.e., 3α/S and 10 α/S equation. Repeatability, intraday and interday precision was performed to assess the level of agreement in the value obtained by proposed method. Repeatability study was performed at concentration 240 and 7992 ng/band of FRF and MMF, respectively after assessing the solution six times in same chromatographic condition, %RSD was computed. At same day and different day precision studies expressed in term of %RSD were performed by analysing three aliquots of 240, 480,720 (ng/band) and 7992, 15984, 23976 (ng/band) of FRF and MMF, respectively in triplicate. The closeness to true value was assessed at 50, 100, 150% level and analysis were performed thrice times for calculating % recovery.

Robustness of the method was evaluated on the basis of slight variation in the mobile phase composition of, amount of methyl alcohol in mobile phase, amount of mobile phase, time required to saturate the chamber, development distance, time period required from chromatography to scanning etc by applying factorial design [FFD],[38,41] five factors i.e., half fractional design (2$^{5-1}$). In the current analysis, five factors were nominated depending on the factor criticality spotted during trial runs and knowledge from the literature and previous studies that are volume of the polar solvent i.e., methyl alcohol in composition of mobile phase (A) volume of mobile phase (B), development distance (C), chamber saturation time (D) time from chromatography to scanning (E). Four critical quality attributes were (CQA) FRF Rf, FRF peak area, MMF Rf, MMF peak area. To examine the deviation quantitatively of the measured response i.e., Rf and peak area of FRF and MMF, the range of factors inspected were intentionally altered from the finalised chromatographic condition. High and low level were set for the mentioned factor by doing the deliberate variation (Table 1). Randomized order was followed to minimize the bias effect of uncontrolled factors of selected variable in experimental domain to perform all trails. After completion of trials robustness of the method was investigated as per the experimental domain by computing the responses such as retention factor and peak area of FRF and MMF.

RESULTS AND DISCUSSION

The migration pattern of the MMF and FRF was studied using single solvents such as methyl alcohol, ethylacetate, chloroform, toluene, acetonitrile, isopropyl alcohol etc on the TLC plates. It was observed that FRF spot migrates with methyl alcohol, isopropyl alcohol only, however, MMF spot migrates with both semi polar and polar solvent. Depending on the migration pattern of FRF and MMF, various solvent system composed of different ratio such as chloroform: ethylacetate: methyl alcohol (6:3:1 v/v/v), toluene: isopropanol: methyl

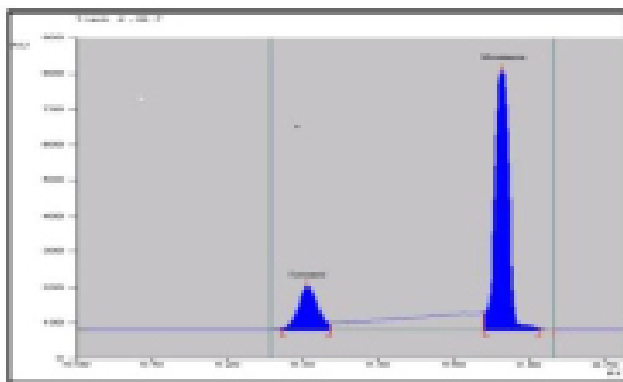| Table 1: Experimental factors and levels used in fractional factorial design.		
Factor	**High Level**	**Low level**
Amount of methyl alcohol in mobile phase, ml [A]	4.2	3.8
Volume of mobile phase, ml (B)	17.62	14.98
Development distance, mm (C)	85	75
Chamber saturation time, minute (D)	25	15
Time from chromatography to scanning, minute (E)	25	15

Figure 2: HPTLC chromatogram of formoterol fumarate (240 ng/band) and mometasone furoate (7992 ng/band).

Table 2: Result of linear regression and validation parameters of FRF and MMF.

Parameters	FRF	MMF
Linearity range (ng/band)	120-720	3996-23976
Correlation coefficient	0.999	0.999
Regression Equation	y = 5.715x + 77.06	y = 0.251x + 7187
LOD (ng/band)	21.943	1160.734
LOQ (ng/band)	66.495	3517.375
Precision (%RSD) Intra-day (*n*=3) Inter-day (*n*=3) Repeatability (*n*=6)	0.974 1.274 0.820	1.050 1.185 0.770
Accuracy (% Recovery studies, *n*=3)		
50	99.762 ± 1.541	100.749 ± 1.598
100	100.972 ± 0.716	100.836 ± 1.585
150	99.235 ± 1.210	101.122 ± 1.260

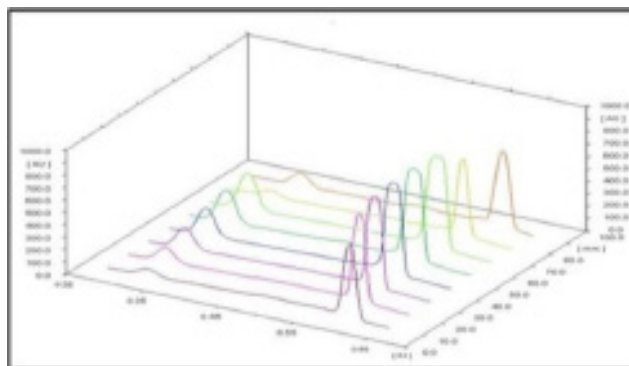*n = number of determinations, % RSD (Percentage relative standard deviation)

Figure 3: Overlain 3D chromatogram of FRF and MMF.

alcohol (6:2:2 v/v/v); toluene: methyl alcohol (8:2 v/v) were tried, but MMF spot migrated near solvent front. Polarity difference of FRF (polar) and MMF (non-polar) is high. So, simultaneous estimation of both the drug with good R_f values was a challenging task. After serval trials mobile phase consisting of toluene: methyl alcohol (12.5:4 v/v) showed acceptable R_f values but the peak symmetry of FRF was not within acceptable range. To correct the peak symmetry of FRF which is weakly acidic drug, methanoic acid, ethanoic acid, triethylamine was tried. Methanoic acid was selected as it improves peak symmetry. So, mobile phase system consisting of toluene: methyl alcohol: methanoic acid (12.5:4:0.3 v/v/v) was selected. A solvent system that gave dense compact spots, good separation between FRF and MMF and also separation from solvent front and application position was selected as shown in Figure 2 with reproducible R_f values 0.316 ± 0.021 and 0.569 ± 0.029 for FRF and MMF, respectively.

The band obtained of FRF and MMF after applying optimised chromatographic condition in the analysis of Evocort® completely match with R_f values and densito-spectra of band of reference drugs. The obtained value of peak purity was near the value of 0.999 defining the specificity of analyse. The concentration of FRF and MMF was found to be directly proportional to its response i.e., peak area in range of 120-720 ng/band and 3996-23976 ng/band, respectively. After applying regression analysis, the calculated value of intercept, slope and correlation coefficient are shown in Table 2 are satisfactory for the method to be linear. The overlay spectra are shown in Figure 3. The values of LOD and LOQ were found to be 21.94, 1160.73 ng/band and 66.49, 3517.37 ng/band for FRF and MMF, respectively representing the determination ability of the method. Results of precision studies was between 0.77-1.274 stated in % RSD indicates good repeatability

and low inter-day variability. Recovery studies ranged from 99-101% are shown in (Table 2) for both the drugs indicating the closeness toward expected value.

Fractional Factorial Design DoE was applied to perform robustness study (Table 3). 2^{5-1} with factors being varied over two levels: fractional and maximum. Four critical quality attributes were (CQA) FRF R*f*, FRF Peak area (PA), MMF R*f*, MMF Peak area (PA) and their obtained value from pareto charts, 3-D response surface plot and perturbation plot are shown in Figure 4a-4f. The desirability value obtained for all CQAs from Pareto charts, 3-D response surface plot, perturbation plot, suggest organic modifier was contributing more in altering response of R*f* of FRF and MMF (Figure 4a-4c). ANOVA equation also shows the same. Mobile phase

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response 1	Response 2	Response 3	Response 4
A: M/P Organic Modifier	B: M/P Volume	C: Development Distance	D: Chamber Saturation Time	E: Time from Chromatography to Scanning	FRF Rf	FRF PA	MMF Rf	MMF PA
3.8	14.98	85	25	25	0.309	1459.43	0.542	9138.67
3.8	17.62	75	15	15	0.309	1498.43	0.548	9279.9
4.2	14.98	75	15	15	0.328	1476.67	0.584	9025.78
4.2	14.98	75	25	25	0.326	1498.8	0.585	9212.62
4.2	14.98	85	15	25	0.328	1498.43	0.582	9046.57
3.8	14.98	75	15	25	0.299	1456.7	0.541	9138.56
3.8	14.98	85	15	15	0.298	1459.44	0.542	9025.78
3.8	17.62	75	25	25	0.309	1476.72	0.548	9267.57
3.8	14.98	75	25	15	0.308	1459.44	0.541	9089.68
4.2	17.62	85	15	15	0.329	1456.72	0.589	9189.62
4.2	17.62	75	15	25	0.329	1476.76	0.589	9138.67
4.2	14.98	85	25	15	0.326	1459.43	0.582	9138.67
3.8	17.62	85	15	25	0.311	1459.43	0.543	9142.64
4.2	17.62	85	25	25	0.329	1459.43	0.589	9138.67
4.2	17.62	75	25	15	0.331	1498.69	0.588	9286.54
3.8	17.62	85	25	15	0.311	1456.72	0.544	9138.67

Table 3: Fractional Factorial Design using factors and found response for robustness evaluation.
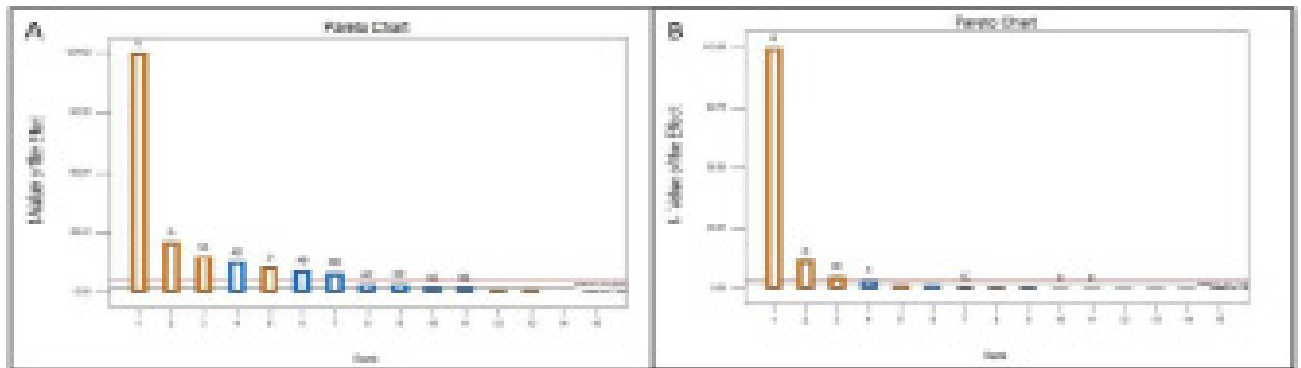

Figure 4a: Pareto chart FRF (A) and MMF (B) showing the effect of factors and interaction on the R_f values.
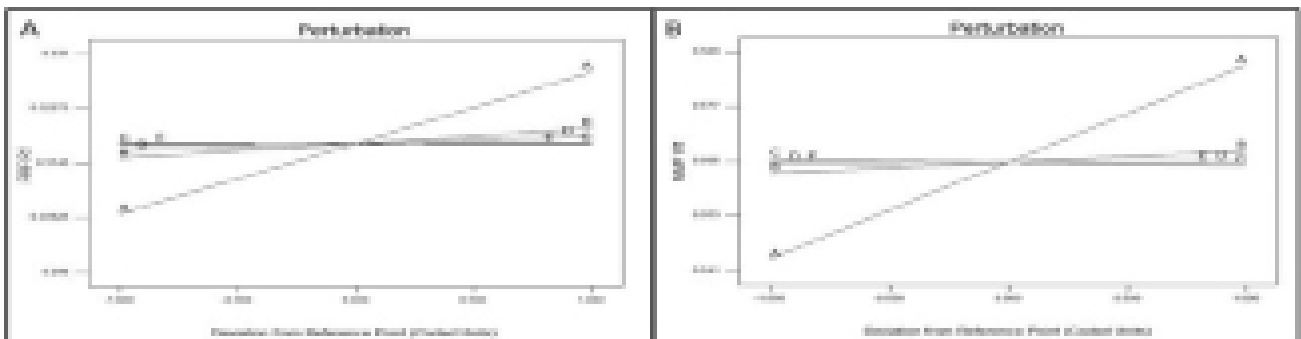

Figure 4b: Perturbation plot of FRF (A) and MMF(B) showing effect of factors on R_f values.
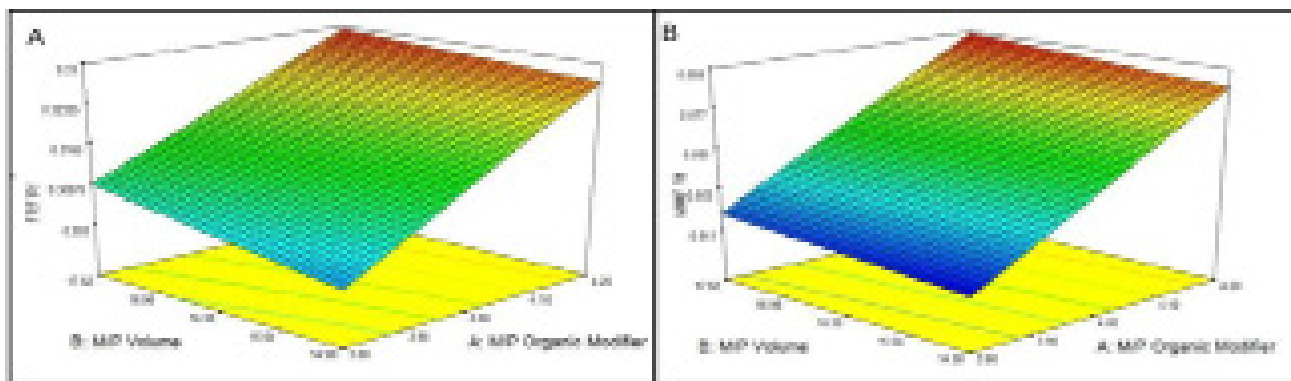
Figure 4c: Three- dimensional response surface plot of FRF (A) and MMF(B) displaying the effect of factors on R*f* values.
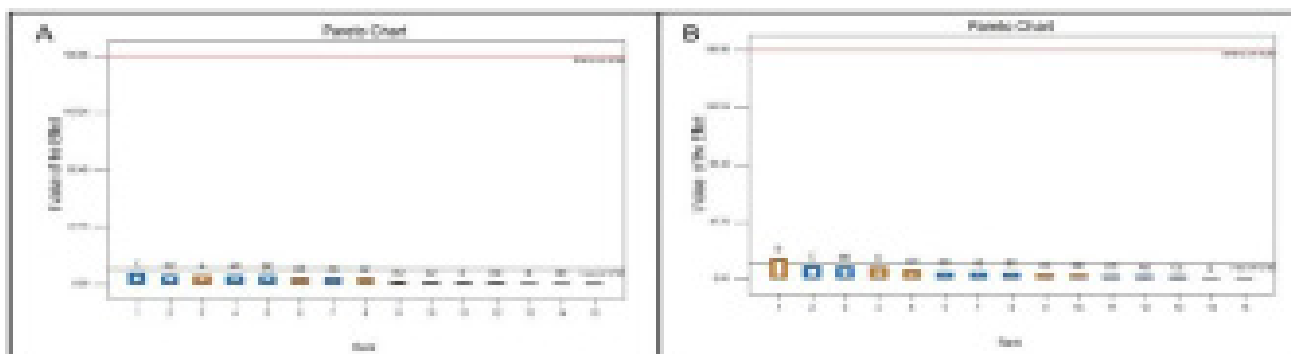

Figure 4d: Pareto chart of FRF(A) and MMF(B) showing the effect of factors and interaction on the peak area values.
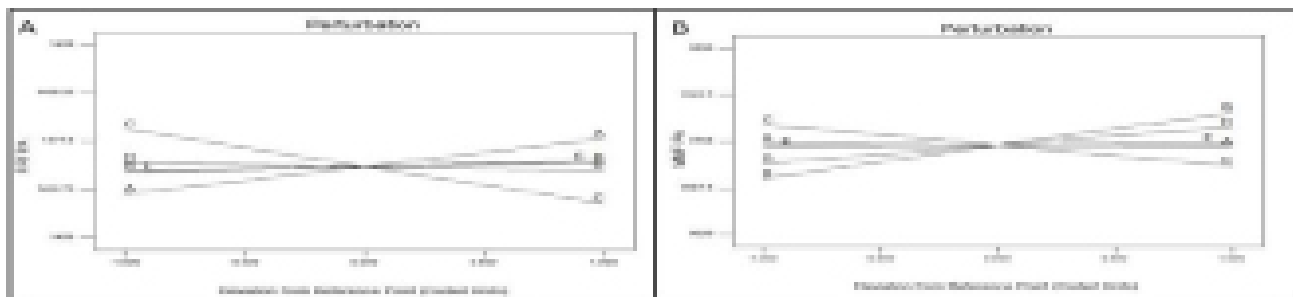

Figure 4e: Perturbation plot showing effect of FRF (A) and MMF(B) factors on peak area values.
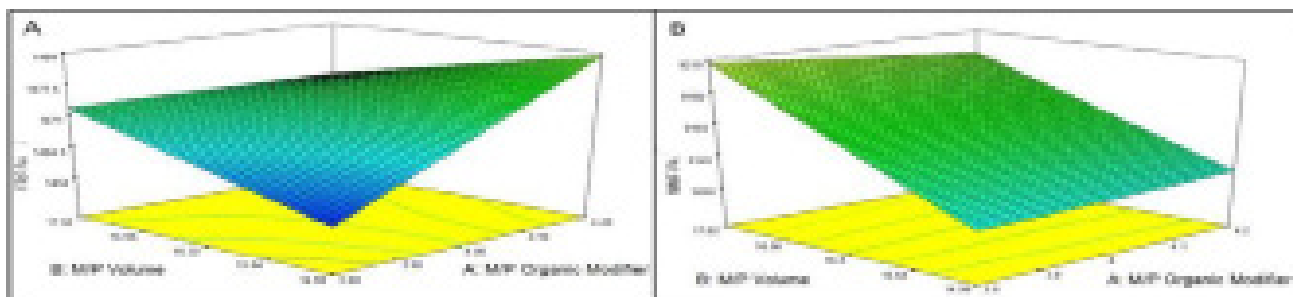

Figure 4f: Three- dimensional response surface plot of FRF (A) and MMF(B) displaying the effect of factors on peak area values.

organic modifier should be controlled. Limit has to be set and strictly controlled for the M/P organic modifier for R*f* of FRF and MMF. Study of peak area of FRF and MMF showed that the method was robust against the evaluated five factors (Figure 4d-4f). Perturbation plots specified that little difference in volume of mobile phase had effects which is vital but unable to show any noteworthy outcome on retention factor except MMF as shown in Figure 4b. Observation can be made from the three-dimensional response surface plots, an increase in concentration of methyl alcohol in the mobile phase result into the slight upward shift of R*f* value of FRF and MMF as shown in Figure 4(c). quations obtained from the model as:

Table 4: Results of Formulation Analysis.			
Drugs	**Amount (µg/rotacaps)**	**% Drug found***	**%RSD**
	Labeled	**Found***		
FRF	6	5.93 ± 0.07	98.94 ± 1.19	1.21
MMF	200	200.94 ± 3.62	100.47 ± 1.81	1.

*Mean ± SD (n=6) values of six determination

FRF R_f = +0.32+0.011 * A+2.250E-003 * B+1.250E-004 * C+1.125E-003 * D+0.000 *

E -1.000E-003 * A * B-3.750E-004 * A * C-1.375E-003 * A * D -2.500E-004 * A * E

-8.750E-004 * B * D-2.500E-004 * B * E+1.625E-003 * C * E -3.750E-004 * D * E

FRF PA =+1471.95+6.16 * A+0.91 * B-8.32 * C-0.87 * D+1.26 * E- 6.13 * A * B

-1.29 * A * C+1.84 * A * D+3.98 * A * E -6.46 * B * C+0.90 * B * D-6.04 * B * E

-4.01 * C * D+4.29 * C * E+1.25 * D * E

MMF R_f=+0.56+0.021 * A+2.437E-003 * B-6.875E-004 * C +6.250E-005 * D

+6.250E-005 * E +1.875E-004 * A * C+1.062E-003 * D * E

MMF PA =+9149.91-2.77 * A+47.87 * B-30.00 * C +26.47 * D+3.08 * E-6.64 * A *

B +11.24 * A * C+20.51 * A * D-16.09 * A * E-15.38 * B * C-16.40 * B * D-28.98 * B *

E -7.71 * C * D-6.36 * C * E+9.91 * D * E

Analysis of formulation

The obtained (% assay) were in the range 98-100 (Table 4). This result suggests that, projected HPTLC investigation was successfully used to find the quantitative amount of FRF and MMF in rotacaps formulation (6 µg of FRF and 200 µg of MMF per rotacaps).

CONCLUSION

The suggested method provide assurance about the sensitivity, effortlessness and specificity for quantitation of the studied drugs in their authentic powders. The Fractional factorial design proposed that the content of methyl alcohol can influence the R_f value of MMF more in comparison to FRF, so it has to be control efficiently for the reproducibility of results. The compatibility of linearity ranges with ratio in their combined rotacaps and also nonappearance of excipients interference recommends application of the proposed methods in quality control analysis of cited drugs in commercial rotacaps.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

FRF: Formoterol Fumarate; **MMF:** Mometasone furoate; **R$_f$:** Retardation factor; **HPTLC:** High performance thin layer chromatography; **RSD:** Relative Standard Deviation; **SD:** Standard deviation; **API:** Active pharmaceutical ingredient.

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PICTORIAL ABSTRACT
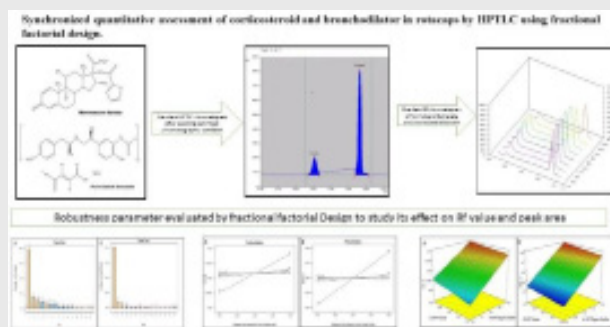
SUMMARY

- The developed and validated HPTLC method was successfully applied for quantification of formoterol fumarate and mometasone furoate by applying DOE. The mobile phase used for the separation of these two analytes was toluene: methyl alcohol: methanoic acid (12.5:4:0.3 v/v/v) on stationary phase of silicagel 60 F_{254}.
- R_f values were found to be 0.316 ± 0.021 and 0.569 ± 0.029 for formoterol fumarate and mometasone furoate, respectively.
- Fractional factorial design was used to examine the effect of multiple robustness parameter efficiently with fewer runs.
- The developed method was successfully validated and therefore can be used in quality control analysis of cited drugs in commercial rotacaps formulation.

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