STABILITY INDICATING HPTLC METHOD FOR ESTIMATION OF BUDESONIDE AND FORMOTEROL FUMARATE DIHYDRATE IN PHARMACEUTICAL FORMULATION

Priyanka S. Shah[a], Chaitali G. Patel[a], Kalpana G. Patel[a]* and Tejal R. Gandhi[b]

(Received 20 April 2019) (Accepted 17 December 2019)

ABSTRACT

A sensitive, selective, precise and accurate stability-indicating high-performance thin layer chromatographic method for analysis of budesonide (BUD) and formoterol fumarate dihydrate (FFD) was developed along with forced degradation study and validated according to ICH guidelines. Densitometry analysis of BUD and FFD was carried out in the absorbance mode at 234 nm using toluene: methanol: ethyl acetate: ammonia (8:2:2.5:0.1,% V/V/V/V) as solvent system. This system was found to give compact spots for BUD at R_f value of 0.34 ± 0.06 and FFD at R_f value of 0.67± 0.05. It was found that besides oxidative, thermal and photo stability studies, acid and base induced degradation of drugs were more with resultant degradation product. 3^2 factorial design was used to predict base induced degradation. The drug undergoes degradation under mainly acidic and basic conditions. Also, the degraded products were well resolved from the pure drugs with significantly different R_f values. Linearity was found to be in the range of 1800-10600 and 1000-6000 ng band^{-1} for BUD and FFD, respectively. The LOQ for BUD and FFD were 392.48 ng band^{-1} and 1189.36 ng band^{-1} and LOD for BUD and FFD was115.79 ng band^{-1} and 350.88 ng band^{-1}, respectively. "Bartlett's test" applied on peak area for linearity, additionally proved validity of the developed method. Good accuracy and precision were obtained as revealed from percentage RSD value less than 2. Similarly, no interference was observed from common excipients in tablet formulation as well as degradation product, indicating specificity of the method. As the method could effectively separate the drug from its degradation product, it can be employed as a stability-indicating one.

Keywords: Budesonide, formoterol fumarate, dihydrate, HPTLC, FFD, Validation, degradation

INTRODUCTION

Budesonide (BUD), chemically mixture of the C*-22*S* (epimer A) and the C*-22*R* (epimer B), epimers of 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene3,20-dione, is an anti–inflammatory corticosteroid[1,2]. Formoterol fumarate dihydrate (FFD), *(2E)*-but-2-enedioic acid bis(*N*-{2-hydroxy-5-[(1*R*)-1-hydroxy-2-{[(2*R*)-1-(4-methoxyphenyl)propan-2-yl]amino}ethyl]phenyl} formamide) dihydrate[3,4] (Fig. 1), is a bronchodilator, adrenergic (inhalation) long-acting β2 agonist (LABA), mainly used for maintenance therapy in patients suffering from asthma and Chronic Obstructive Pulmonary Disease (COPD)[5].

Literature reports the analysis of BUD and FFD by high performance liquid chromatography and UV spectrophotometric methods[6–10]. RP HPLC, UV and HPTLC are also reported individually for analysis of BUD and FFD as well as with other drugs[11-24]. Moreover, literature reviewed reveals no information related to the stability-indicating methodology by high performance thin layer chromatography (HPTLC) for the determination of BUD and FFD in pharmaceutical dosage forms. Accordingly, the purpose of the present study was to put ICH recommendations into practice by subjecting BUD and FFD to a variety of suggested stress test conditions study and evaluate stability of the drug and to develop a validated stability indicating HPTLC assay.

TLC and HPTLC techniques are rapidly becoming adaptable due to their various advantages over other methods[25]. The various advantages of HPTLC includes, more number of samples can be run simultaneously using less amount of mobile phase unlike HPLC, thus lowering the time required for analysis, sample clean up and overall cost per analysis[26]. Hence, separation and quantification

[a] Pharmaceutical Quality Assurance Department, Anand Pharmacy College, Opposite Town Hall, Anand - 388 001, Gujarat, India
[b] Pharmacology Department, Anand Pharmacy College, Opposite Town Hall, Anand - 388 001, Gujarat, India
*For Correspondence: E-mail: kalpana_jpatel@yahoo.com

https://doi.org/10.53879/id.58.12.11830

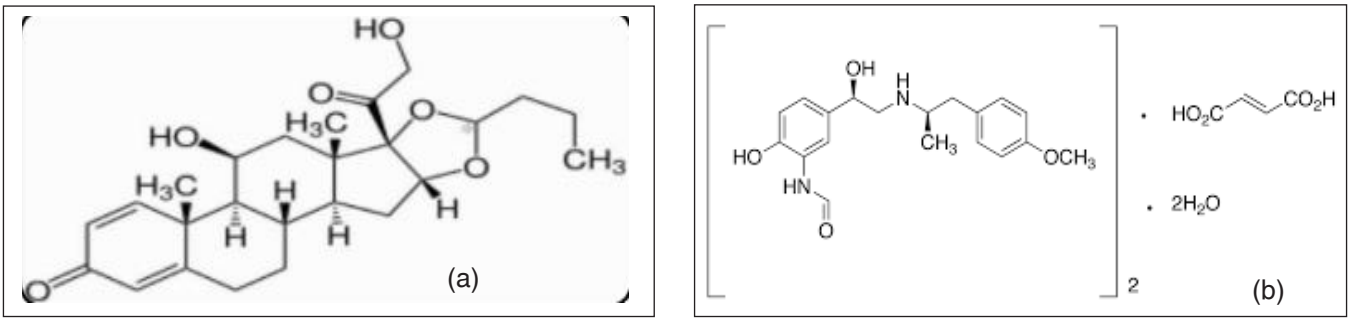

Fig. 1: Chemical structures of (a) budesonide and (b) formoterol fumarate dihydrate

can provide results that are either superior or comparable with other analytical methods such as HPLC[27]. A stability indicating method (SIM) is an analytical procedure used to quantitate the decrease in the amount of drug substance in drug product due to degradation as per ICH[28]. Hence, stress testing can aid in identifying degradation products resulting in information about the intrinsic stability of drug[29].

This research paper describes the development of HPTLC method for simultaneous estimation of BUD and FFD using AQbD and Design of Experiment approach for Stability indicating method. AQbD is a science and risk-based paradigm for analytical method development, endeavoring for understanding the predefined objectives to control the critical method variables affecting the critical method attributes to achieve enhanced method performance, enhanced method control, high robustness, ruggedness and flexibility for continual improvement resulting in smooth process of method transfer to the production level[30]. A stability indicating method was developed by HPTLC that is capable of quantifying and can also resolve BUD and FFD from its degradation products. The proposed stability indicating method is simple and allows rapid analysis for stability studies and quality control analysis of drug in bulk and dosage form[31].

MATERIALS AND METHODS

Materials

BUD and FFD of pharmaceutical grade was obtained as gratis samples from Sun Pharmaceutical Industries Limited, Vadodara, Gujarat. The formulation used was an inhaler containing labeled claim 80 µg of BUD and 4.5 µg of FFD per actuation from Astra Zeneca. All chemicals and reagents used were procured from S.D Fine Chemical Ltd., Mumbai and of analytical reagent grade.

Instrumentation

Linomat 5 applicator (CAMAG, Switzerland), Microsyringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, CAMAG, Switzerland), twin trough chamber (10·20 cm; CAMAG, Switzerland), UV chamber (CAMAG, Switzerland), TLC scanner IV (Camag, Switzerland), pre-coated silica gel $60F_{254}$ aluminum plates (10·10 cm, 100 µm thickness; Merck, Darmstadt, Germany), winCATS version 1.4.6 software (CAMAG, Switzerland) were used in the study.

Preparation of standard stock solution

Stock solution of BUD and FFD were prepared by weighing accurately 10 mg of drug followed by dissolution in methanol in 10 mL volumetric flask and dilution up to the mark with methanol, to obtain a concentration of 1000 µg mL^{-1}. These standard stock solutions were used as working standard solution.

Chromatographic procedure

The samples were spotted in the form of bands having band width 6 mm with a 100 microlitre microsyringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz, CAMAG, Switzerland) on precoated silica gel aluminum HPTLC Plate $60F_{254}$, (20 cm ×10 cm), 100 µm thickness; (E. Merck, Darmstadt, Germany) using a CAMAG Linomat V sample applicator (Switzerland). Linear ascending development was carried out in 20 ×10 cm twin trough glass chamber (CAMAG, Switzerland). The mobile phase consisted of toluene: methanol: ethyl acetate: ammonia (8:2:2.5:0.1, %V/V/V/V). The optimized chamber saturation time before chromatographic development was 20 min at room temperature (25 °C±2 °C). The length of chromatographic run was 8 cm. Subsequent to the development; HPTLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner IV with winCATS software (Design Expert trial version 7.0.0). All measurements were made in the reflectance absorbance mode at 234 nm, slit dimension (6.00x0.30 mm, micro), scanning speed 20 mm s^{-1}, data resolution 100 µm $step^{-1}$, optical filter (second order), filter factor (Savitskygolay 7). The source of radiation was deuterium lamp emitting a continuous UV spectrum between 200 and 700 nm.

Calibration curve

Different volumes of standard stock solution were applied and linear relationship between peak area and concentration of the drugs was evaluated over the concentration range, expressed in ng band^{-1}, by making five replicate measurements of 1000-6000 ng band^{-1} of FFD and 1770- 10662 ng band^{-1} of BUD. Calibration plots were constructed by plotting the peak area of the main band versus the concentration of the drug. Evaluation was via peak areas with linear regression analysis.

METHOD VALIDATION

The method was validated in accordance with ICH guidelines Q2 (R1) for evaluation of linearity, precision, accuracy, LOD, LOQ, specificity and robustness[32].

Precision

Precision of the developed method was evaluated by performing repeatability on same day and intermediate precision studies on different days and peak area measured was expressed in terms of percent relative standard deviation (%RSD). Repeatability was carried out by performing three replicates of three different concentration (1800, 5300 and 10600 ng band^{-1} of BUD and 1000, 3000 and 5000 ng band^{-1} of FFD) on the same day and peak area measured was expressed in terms of percent relative standard deviation (%R.S.D.). Similarly, intermediate precision study was performed on different days.

Accuracy

Accuracy of method was ascertained by performing recovery at three levels (50, 100 and 150 %). Recovery studies were carried out by spiking three different amount of BUD standard (1800, 3600 and 5400 ng band^{-1}) to the dosage form (3600 ng band^{-1}) by standard addition method. Similarly, recovery studies were carried out by spiking three different amounts of FFD standard (1000, 2000 and 3000 ng band^{-1}) to the dosage form (2000 ng band^{-1}) by standard addition method. Recovery studies were performed in triplicate.

Limit of detection (LOD) and limit of quantitation (LOQ)

As per ICH guidelines, limits of detection and quantification of the developed method were calculated from the standard deviation of the y-intercept and slope of the calibration curve of BUD and FFD using the formula:

Limit of detection=$3.3*\sigma/S$

Limit of quantitation=$10*\sigma/S$

where "σ" is SD of intercept

"S" is Slope of calibration curve

Specificity

The specificity of the method was ascertained by analyzing peak purity of standard drug and sample. The spot for BUD and FFD in sample and degradation studies was confirmed by comparing the R_f values and spectra of the spot with that of standard. The peak purity of BUD and FFD was assessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) of the spot.

Robustness

As defined by the ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters. Here changes in different conditions were considered: Mobile phase ratio (2.5 ± 0.1 mL for one component), saturation time (20 ± 1 min), distance travel (8 cm ± 2 mm) and wavelength change (234 ± 2 nm) were studied in terms of R_f and peak area and expressed as % RSD.

Analysis of marketed formulation

Inhalation powder was taken through actuation from the canisters which contain 4.5 µg FFD and 80 µg BUD. 50-actuations equivalent to 225 µg of FFD and 4000 µg of BUD was transferred to mortar pestle and triturated to convert into powder from. The powder was dissolved in 5 mL methanol and ultra-sonicated for 15 min. The resultant solution was filtered through Whatman filter paper (No. 42). 10 µL of resultant solution of BUD (8000 ng band^{-1}) or 25 µL of resultant solution of FFD (1125 ng band^{-1}) was applied to HPTLC plate and the plate was run under optimized chromatographic condition using detection wavelength 234 nm.

FORCED DEGRADATION STUDIES

To evaluate the stability indicating property of the developed HPTLC method, standard drug was subjected to forced degradation conditions like acid/base hydrolysis, oxidation and photo degradation. In all degradation studies, area % of BUD and FFD and degradation product was measured for calculation. The forced degradation study in acid, base and hydrogen peroxide was performed in the dark, in order to exclude the possible degradative effect of light on the drugs[33-36].

Acid and base induced degradation study

Acid induced degradation was attempted by taking 1 mL of a standard solution of BUD (10000 µg mL^{-1}) and FFD (1000 µg mL^{-1}) with 1 mL of hydrochloric acid (0.01 M, 0.02 M and 0.05 M, separately) in volumetric flask. These solutions were kept for 30 min at room temperature in order to exclude the possible degradative effect. The resultant solution was neutralized with 1 mL of sodium hydroxide (0.01 M, 0.02 M and 0.05 M, separately) and diluted upto 10 mL with methanol to obtain final concentration of BUD (1000 µg mL^{-1}) and FFD (100 µg mL^{-1}). 10 µL of BUD (10000 ng $band^{-1}$) and 15 µL of FFD (1500 ng $band^{-1}$) samples were directly applied to HPTLC plates and the chromatograms were run under optimized chromatographic conditions. Similarly, base induced degradation was performed like the above method. 3^2 factorial design was taken for base induce degradation. Three levels were chosen for both factors; Sodium hydroxide concentration and different time duration (min). 2 variables were considered at three levels (Table I).

Table I : Experimental domain with actual values for factor levels using 3^2 factorial design for base induced degradation

Runs	Factor A	Factor B
	Concentration (M)	Time (min)
1	0.03	10
2	0.01	10
3	0.02	10
4	0.02	20
5	0.02	20
6	0.01	30
7	0.02	20
8	0.02	20
9	0.03	20
10	0.01	20
11	0.02	30
12	0.03	30
13	0.02	20

Hydrogen peroxide-induced degradation study

For oxidative induced degradation, experiment was performed by transferring 1 mL of a stock standard solution (BUD, FFD) to 10 mL volumetric flask individually followed by adding of 1mL of hydrogen peroxide (0.3 %V/V and 3 %V/V H_2O_2, separately). Solutions were kept for 30 min and diluted upto mark with methanol to obtain final concentration of BUD 1000 µg mL^{-1} and for FFD 100 µg mL^{-1}. 10 µL of BUD (10000 ng $band^{-1}$) and 15 µL of FFD (1500 ng $band^{-1}$) samples were directly applied to HPTLC plates and the chromatograms were run under optimized chromatographic conditions.

Photo-degradation study

For the photo-degradation study, standard powder was separately exposed to UV light (in a UV chamber) and sunlight for 24 h. After that, appropriate dilutions were made in methanol to obtain final concentration of 100 µg mL^{-1} and 1000 µg mL^{-1} for FFD and BUD, respectively. 10 µL of BUD (10000 ng $band^{-1}$) and 15 µL of FFD (1500 ng $band^{-1}$) samples were directly applied to HPTLC plates and then run under optimized chromatographic conditions.

Thermal-degradation study (dry heat)

For dry heat degradation, BUD and FFD powder was placed in an oven at 70 °C and 80 °C for 30 min. Thereafter, drug powder was diluted to 10 mL with methanol and samples were directly applied to HPTLC plates and the plates were run under optimized chromatographic conditions.

Statistical analysis

Statistical parameters like SD and % RSD were computed using MS Excel. Bartlett's test and test for lack of fit were applied on the data of areas of linearity for evaluation of homoscedasticity of variance and deviation from linearity[37]. The software Design Expert used generated model (mathematical equation) based on the factors and responses, along with graphical representation providing correlation between factors and response.

RESULTS AND DISCUSSION

Optimization of mobile phase

In order to develop stability indicating method, pure drug and its degradation product were applied on plates and different solvents, alone and its combinations were tried in different ratios using methanol, toluene, ethyl acetate, trimethylamine and ammonia. Good and sharp peak was obtained in optimized mobile phase toluene: methanol: ethyl acetate: ammonia (8:2:2.5:0.1, % V/V/V/V), (Fig. 2) with R_f of BUD 0.34 ± 0.06 and FFD 0.67± 0.05 with degradation product peak separated from the drug peak. Sharp and well-defined symmetrical peaks were obtained when the chamber was saturated with mobile phase for 30 min at room temperature and scanned at 234 nm.

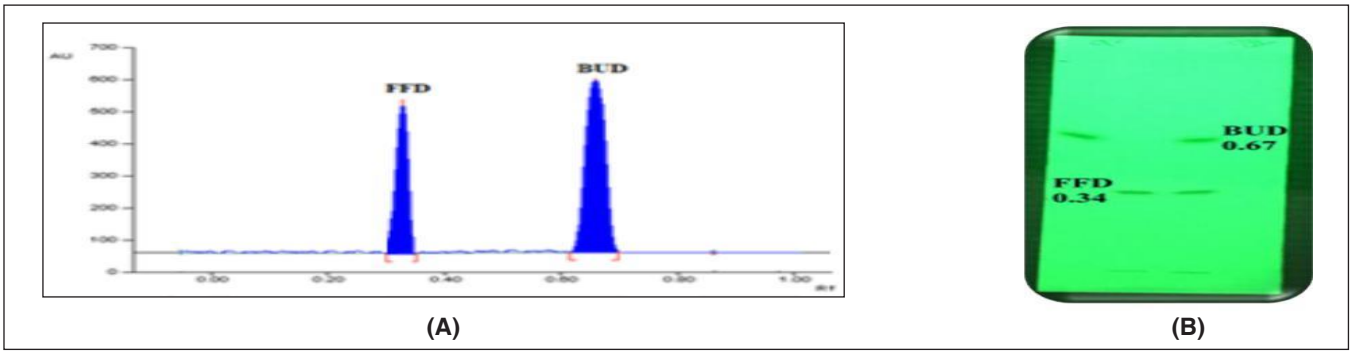

Fig. 2: (A) Chromatograms of BUD and FFD standard (B) TLC image of FFD and BUD standard

VALIDATION OF METHOD

The method was validated as per ICH guidelines.

Linearity

BUD and FFD showed good correlation over the concentration range of 1800-10600 ng band^{-1} and 1000-6000 ng band^{-1} with respect to peak area, respectively. The linearity of calibration curve and adherence of system to Beer's law were evaluated by high value of correlation coefficient. Further linearity was validated by ''Bartlett's test'' confirming homoscedasticity of variance that was exemplified by $\chi 2$ value less than the tabulated value (Table II).

Precision

% Relative standard deviation for repeatability of sample application and intermediate precision was found to be less than 2 for BUD and for FFD, showing good method precision. % RSD value reveals that the proposed method provides acceptable precision of the method.

Accuracy

The proposed method when evaluated for accuracy in terms of percent recovery at three levels (50, 100 and 150 %), showed percentage recovery at all three levels in the range of 97.80-98.26 for BUD and 99.99-99.39 for FFD, suggesting suitability of the method to perform routine drug analysis (Table II).

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD of FFD and BUD was found to be 393.107 and 392.489 ng band^{-1}, respectively while LOQ of FFD and BUD was found to be 1191.233 and 1189.361 ng band^{-1} for the proposed method, indicating good sensitivity of the method.

Table II: Summary of validation parameters for BUD and FFD

Parameter	BUD	FFD
Calibration range[a] (ng band^{-1})	1800-10600	1000-6000
Regression equation	y = 2.0747x + 8388.4	y = 1.7237x + 4374.5
Regression coefficient	0.9989	0.9984
Correlation coefficient	0.9994	0.9992
Standard deviation of slope	0.05194	0.02945
Confidence limit of slope[b]	2.00-2.145	1.689-1.768
Standard deviation of intercept	246.759	60.509
Confidence limit of intercept[b]	8074.6-8785.8	4284.6-4454.4
Limit of detection (ng band^{-1})	392.489	115.791
Limit of quantification (ng band^{-1})	1189.361	350.882
Bartlett's test ($\chi 2$)	0.016841689	0.0044
Precision (%RSD)[d] Repeatability	1.046-1.102	0.715-1.227
Intermediate precision	1.104- 1.673	0.982-1.691
Accuracy (% recovery)[e]	97.80-98.26	99.99 -99.39

[a]mean of five replicates ,[b]Calculated value $\chi 2$ less than critical value $\chi 2(0.05, 4)=9.488$, [c]Confidence interval at 95 % confidence level and four degree of freedom (t=2.13) Calculated F value less than tabulated value, 5.92 at 95% confidence interval; [d]Average of three determinations for each concentration; [e]Average of three determinations at each level

Specificity

The chromatogram of the formulation obtained using the developed method showed peak at R_f value of 0.67 and 0.32 for BUD and FFD, respectively, and was found to be at the same R_f value for both standard drugs by comparison of chromatograms (Fig. 3). The peak purity of both drugs in pharmaceutical dosage form when evaluated by comparing the overlain spectra at peak start, peak apex and peak end positions of the spot, indicated that purity was more than 0.99 for all peaks, indicating specificity in presence of excipients.

Robustness

A deliberate change in different parameters like mobile phase composition, chamber saturation time, distance travel and wavelength showed that there was no significant change in the R_f and peak area, indicating that the method is robust.

Analysis of marketed formulation

The marketed formulation containing 80 µg BUD and 4.5 µg FFD when analyzed in triplicate using the developed method, showed separate peak at R_f value of 0.34 for FFD and 0.67 for BUD and mean percent recovery was found to be 97.92 and 98.50 % for BUD and FFD, respectively, with no interference of the excipient (Table IV).

Table III: Statistical parameter from ANOVA

Parameter	% Degradation of BUD	% Degradation of FFD
Standard deviation	1.08	2.09
Mean	13.29	21.62
CV(%)	8.12	9.68
PRESS	65.57	105.52
R-Squared	0.9862	0.9645
Adjusted R-Square	0.9764	0.9574
Predicted R-Square	0.8893	0.9145
Adequate precision	35.532	38.691
Polynomial equation	+14.35+7.55*A +5.48*B +3.53*A*B - 1.49*A2 -0.80*B2	-17.29564 +759.00000*A +1.18667*B

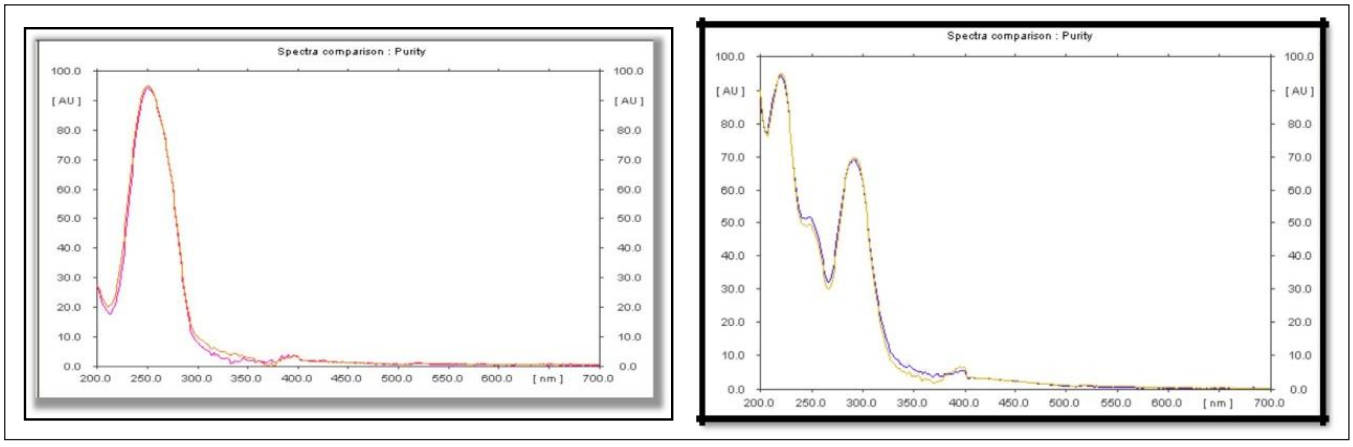

Fig. 3: Overlain spectra of pharmaceutical tablet formulations with standard showing peak purity

Forced degradation study

The suitability of the proposed method for estimation of FFD and BUD in the presence of its degradation product was confirmed by performing forced degradation study under various conditions. The content of FFD and BUD remaining in terms of % degradation was calculated. The recovery in 0.05 M hydrochloric acid, 70 °C, 15 min was found to be 15.10 % for FFD and 23.97 % for BUD respectively. Similarly, % degradation in 0.05 M sodium hydroxide, 70 °C, 15 min was found to be 17.11 % for FFD and 27.11 % for BUD respectively. This difference in drug degradation indicates that alkaline degradation is slightly more pronounced than acid degradation. Acid induced degradation of BUD (0.05 M HCl at 70 °C for 15 min) resulted in R_f 0.41 and R_f of degradation products of BUD at 0.20, 0.25, 0.27, 0.41, 0.50. Similarly base induced degradation of BUD (0.05 M NaOH at 70 °C for

Table IV: Analysis of marketed formulation

Drug	Label Claim	Concentration (ng band^{-1})	% Recovery			Mean% recovery[a]	%RSD
			1	2	3		
BUD	80 mg	8000	97.86	97.28	98.61	97.92	0.557
FFD	4.5 mg	1125	98.58	99.73	97.19	98.50	1.055

[a]mean of three replicates, % RSD= Relative standard deviation

15 min) gave R_f 0.66, and R_f of degradation products of BUD at 0.44, 0.48, 0.75. In case of oxidation by hydrogen peroxide in 0.3 % and 3 % separately at room temperature, resulted in degradation with degradation product peak at 0.51 for FFD and 0.24, 0.40, 0.57, 0.72 for 3% hydrogen peroxide at the 30 min was observed. Moreover, drug was exposed to UV and sunlight. Additional peak at R_f value of 0.45 for FFD and 0.67 for BUD was observed in UV at 24 h at 254 nm. Thermal degradation at 70 °C and 80 °C showed very less degradation for FFD and BUD (Fig. 4).

Full factorial design for base induced degradation Study

FFD and BUD were found to undergo base induced degradation in 0.01 M, 0.02 M and 0.03 M NaOH. The degradation in 0.01 M NaOH at 70 °C when refluxed for 10 min showed less degradation compared to 0.03M NaOH. The peaks of degradation product (DP) of FFD were found at R_f values of 0.20, 0.22, 0.43, 0.45, 0.47, 0.79 while the peaks of degradation product (DP) of BUD were found at R_f values of 0.21, 0.40,0.44, 0.70, 0.76.

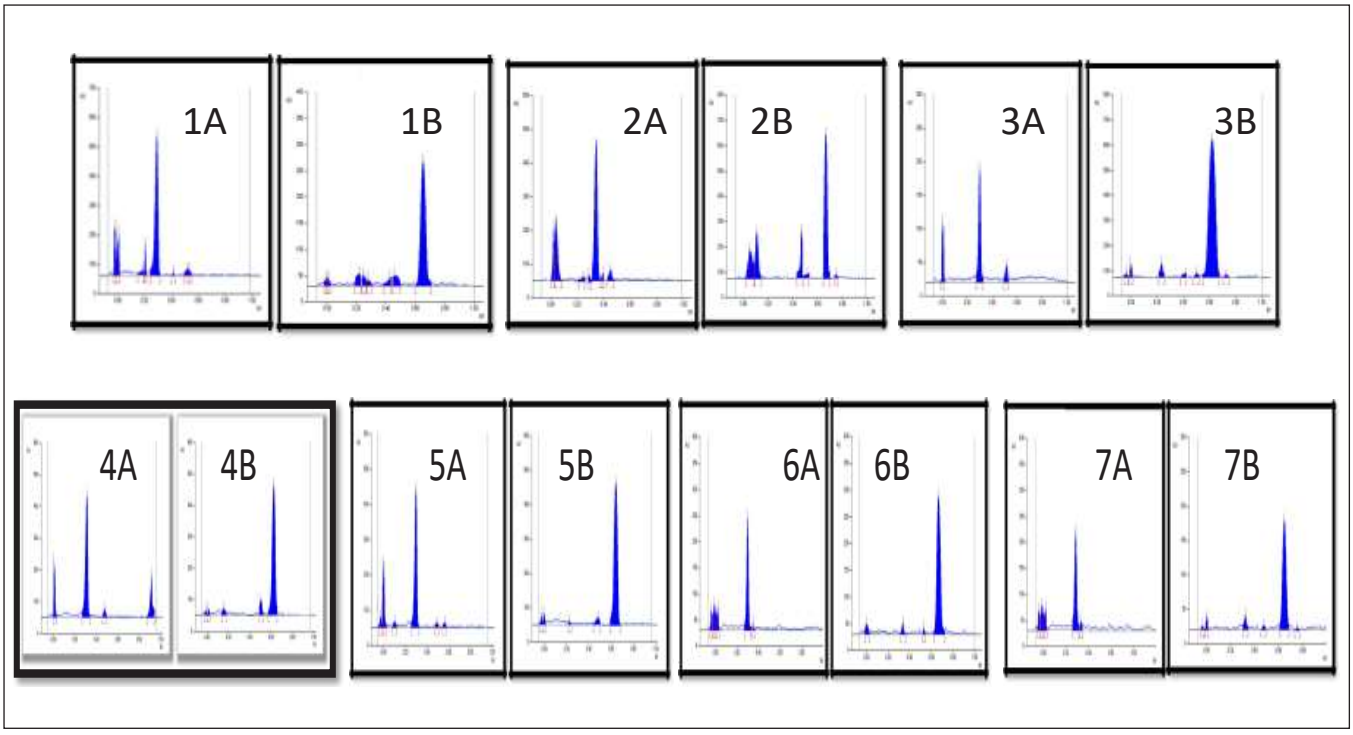

Fig. 4: Degradation Study of (A) BUD and (B) FFD at various conditions, (1A and 1B) Acid induced degradation (0.05 M HCl at 70 °C for 15 min); (2A and 2B) Base induced degradation (0.05 M NaOH at 70 °C for 15 min); (3A and 3B) 3 %V/V H_2O_2 degradation for 30 min; (4A and 4B) UV light induced degradation by exposure for 24 h at 254 nm; (5A and 5B) Sun light induced degradation by exposure for 24 h; (6A and 6B) Thermal degradation at 70 °C; (7A and 7B) Thermal degradation at 80 °C

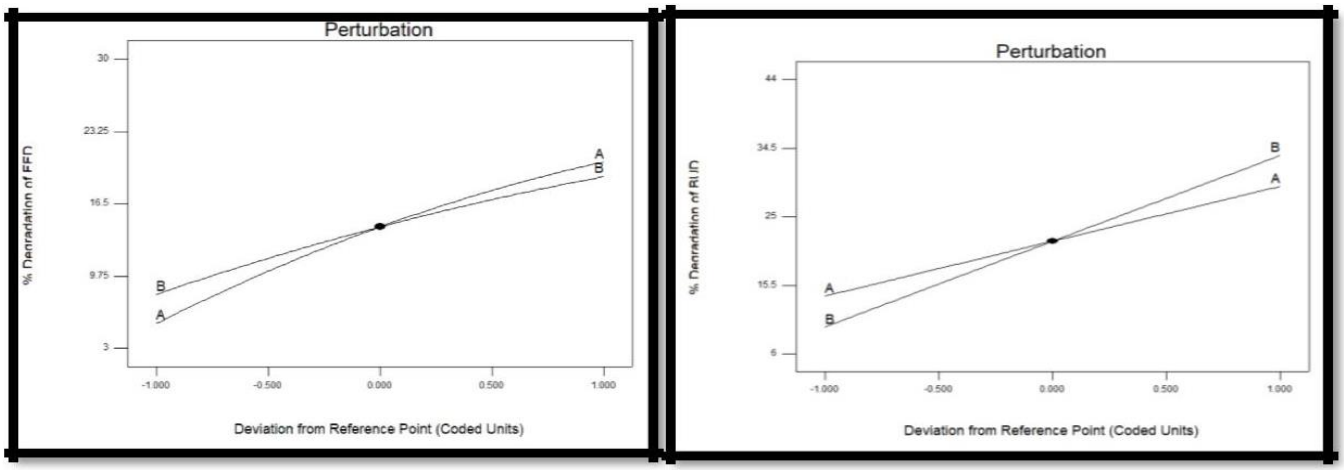

Fig. 5: Perturbation graph showing the effect of each factor A, B, on % degradation of both drugs BUD and FFD

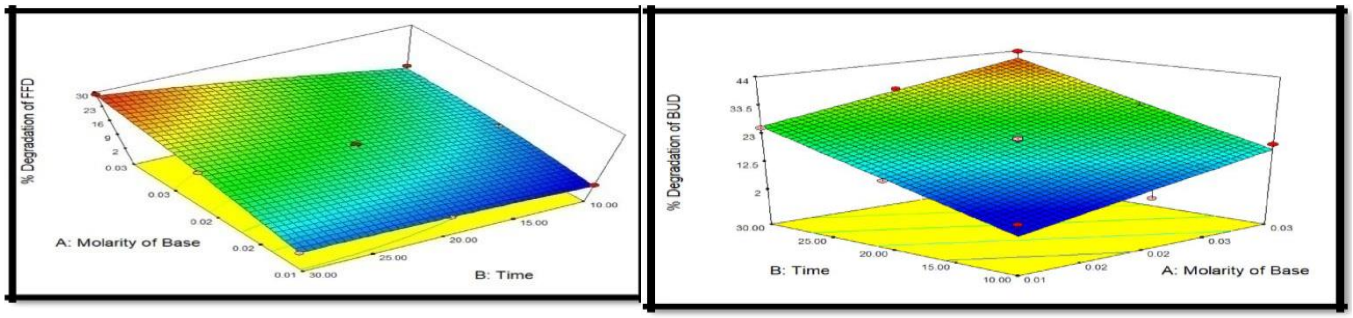

Fig. 6:Three-dimensional response surface plot for % degradation of FFD and BUD

The statistics parameter for each response is shown in the Table III. The difference observed in the predicted and adjusted R – square is less, depicting close agreement. In the present study, the value of adequate precision, (depicts the value of signal to noise ratio) greater than 4 % is desirable, the value coefficient of variation (CV) (measures the reproducibility of the model) less than 10 % is desirable and the P-value of the models ($p < 0.05$ is required) these all were in desirable limits (Table III). It reveals that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors, showing a good agreement between experimental and predicted values.

Here, predicted models are presented in the form of perturbation plots for better understanding of results (Fig. 4). These graphs give the idea about how the response changes as each factor moves from its defined reference value, with all other factors held constant. A steep slope or curvature in a factor indicates that the response is sensitive to that factor. It is evident from Fig. 5 that factors time (B) and concentration (A) shows higher degradation of BUD and FFD when the levels are increased from lower to higher levels.

The representative plots for response R1 are presented in Fig. 6, (B) and showing the interaction between the variables (factors (A) molarity of Base, (B) time) and their mutual dependence can be clearly observed. The % degradation of FFD was increasing as the concentration and time increases (Fig. 6). The representative plots for response R2 are presented in Fig. 6, (B) and showing the interaction between the variables (factors (A) molarity of base, (B) Time) and their mutual dependence can be clearly observed.

CONCLUSION

A specific, precise, accurate, rapid and economical stability indicating high-performance thin layer chromatographic method for analysis of budesonide (BUD) and formoterol fumarate dihydrate (FFD) in bulk drug and marketed formulation is developed and validated according to ICH guidelines. Developed HPTLC method shows various advantages like, several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering the analysis time, sample clean up and cost per analysis. The developed method could effectively separate the drugs from its degradation

products; hence it can be employed as a stability-indicating one. Also, it is suitable for the analysis of BUD and FFD in bulk and dosage form.

ACKNOWLEDGEMENTS

The authors express their gratitude to Sun Pharmaceutical Industries Limited, Vadodara, Gujarat for providing gift sample of standard budesonide and formoterol fumarate dihydrate.

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