A Novel Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Beclomethasone Dipropionate and Fluconazole in Pharmaceutical Dosage Form

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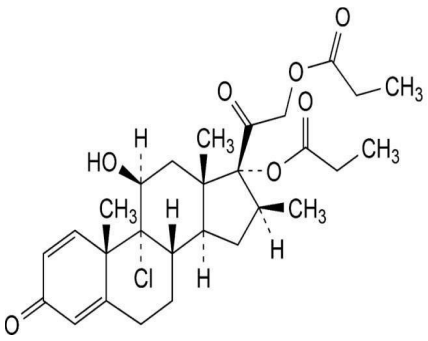
Abstract

The objective of the present research work was to develop and validate stability indicating RP-HPLC method for the simultaneous estimation of Beclomethasone dipropionate and Fluconazole in Pharmaceutical dosage form as per ICH guidelines. The separation was achieved on a reverse phase column BDS Hypersil C18 (250 x 4.6mm, 5 µm) with mobile phase comprising of phosphate buffer 0.05 M (pH 4.0): Methanol (65:35%v/v) and the eluents were detected at 220nm. The retention time of Beclomethasone dipropionate and Fluconazole was found to be 7.407min and 4.067 min, respectively with the flow rate of 1 ml/min. The column temperature was maintained at 26^{o}C. Linearity observed for Beclomethasone dipropionate 0.5-1.5µg/ml and 40-120µg/ml for Fluconazole. The correlation coefficient was found to be 0.9995 for the both drugs. Recoveries ranged in between 98-102%. The low % RSD values indicate the method to be accurate and precise. The LOD and LOQ for Beclomethasone dipropionate were found to be 0.033µg/ml and 0.100µg/ml, respectively. The LOD and LOQ for Fluconazole were found to be 2.788µg/ml and 8.448µg/ml, respectively. The method validation parameters were performed as per ICH Q2R1 guidelines. Drugs were subjected to stress conditions of acidic, basic, oxidative, photolytic and thermal degradation. The developed method was found to be statically validated, simple, rapid, specific, sensitive, accurate, economical and precise. The degraded products were well resolved from pure drugs with significantly different retention time values. No interference from excipients and degradants products was found. The proposed method successfully applied for estimation of Beclomethasone dipropionate and Fluconazole in Pharmaceutical dosage form.

Keywords: *Beclomethasone dipropionate, Fluconazole, Stability indicating RP – HPLC method, Forced degradation, Validation*

1. Introduction

Beclomethasone dipropionate is chemically known as[2-[(8S,9R,10S,11S,13S,14S,16S,17R) - 9 - chloro - 11 - hydroxyl - 10, 13, 16 - trimethyl - 3 - oxo - 17 - propanoyloxy - 6,7,8,11,12,14,15,16 octahydrocyclopenta [a] phenanthren - 17 - yl] - 2 - oxoethyl] propanoate. Beclomethasone dipropionate is a potent gluco-corticoid steroid. It is a pro-drug of the free form Beclomethasone. It is used in prophylaxis of asthma and anti-rhinitis. [1-5]



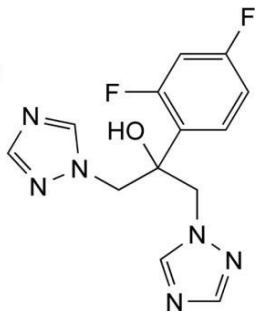


Figure 1. Structure of Beclomethasone Dipropionate Figure 2. Structure of Fluconazole

Fluconazole is chemically known as 2 - (2, 4 - difluorophenyl) - 1, 3 - bis (1H - 1, 2, 4 - triazolo - 1- yl) propan - 2 - ol. Fluconazole is a Triazole antifungal agent that is used to treat or pharyngeal candidiasis and cryptococcal meningitis in AIDS. [1- 4]

Extensive literature review revealed that Beclomethasone Dipropionate and Fluconazole are official in IP, USP30-NF27 and BP. There was several HPLC methods reported officially in IP, BP and USP for estimation of Beclomethasone Dipropionate and Fluconazole in single dosage form. Also, stability indicating RP-HPLC, UV, HPLC, HPTLC and LC-MS methods were reported for estimation of Beclomethasone Dipropionate and Fluconazole in single and combined dosage form with other drugs and in biological fluids. There was no reported method available for simultaneous estimation for Beclomethasone Dipropionate and Fluconazole in combined pharmaceutical formulations. [2-10]

The aim of present work was to develop and validate novel, simple, specific and sensitive stability indicating reverse phase high performance liquid chromatography for simultaneous estimation for Beclomethasone Dipropionate and Fluconazole in combined pharmaceutical formulations as per ICH guidelines.

2. Materials and Methods

Instrumentation

HPLC system used was an Agilent Technologies LC System equipped with SPD 20A UV Detector, Data were collected and processed using Spinchrom software from Agilent, Manual injector of 20-µl loop, Column – BDS Hypersil C18 (250mm×4.6 mm i.d.,5µm), Digital pH meter (Analab Scientific instruments Pvt. Ltd.) were also use.

Chemicals and reagents

The Pure Beclomethasone Dipropionate and Fluconazole were provided by Remus Remedies as a gift sample. The solvents used were HPLC grade and chemicals used were analytical grade. Methanol and water (HPLC grade) were purchased from Merck Chemical Company. Potassium dihydrogen orthophosphate, Triethylamine (Analytical grade) and 0.22 µm pump Nylon filter were purchased from S.D. Fine Chemicals Ltd., Mumbai.

Chromatographic conditions

Drugs were analyzed using a BDS Hypersil C18 column (250mm × 4.6mm, 5µ). The Mobile phase was a mixture of phosphate buffer 0.05 M (pH 4.0): Methanol (65:35%v/v). The Mobile phase was filtered through 0.22 µm Nylon filter and degassed by ultra sonicator. A flow rate of 1.0 ml/min with an injection volume of 20 µl and detection wavelength of 220 nm was used. [11]

Preparation of standard stock solutions

Preparation of Fluconazole standard stock solution (800µg/ml)

80 mg of Fluconazole was weighed and transferred to a 100ml volumetric flask. Volume was made up to the mark with Methanol.

Preparation of Beclomethasone dipropionate standard stock solution (10µg/ml)

10 mg of Beclomethasone dipropionate was weighed and transferred to a 100ml volumetric flask. Volume was made up to the mark with Methanol and from this solution pipetted out 10 ml and transferred to a 100 ml volumetric flask and volume was made up to the mark with Methanol.

Preparation of standard solution of binary mixtures of Fluconazole (80µg/ml) and Beclomethasone dipropionate (1µg/ml)

1 ml from the Fluconazole stock solution and 1ml from Beclomethasone dipropionate stock solution was transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

Preparation of calibration curves

Calibration solutions of concentration ranges 40-120 µg/ml (Fluconazole) and 0.5-1.5 µg/ml (Beclomethasone Dipropionate) were prepared by proper dilution of standard stock solution. From standard stock solution of Fluconazole (800µg/ml) and Beclomethasone Dipropionate (10 µg/ml) aliquots (5,7.5,10,12.5,15 ml) of solutions were pipetted out and transferred to 100 ml Volumetric flask and make up with mobile phase. The graph of peak area obtained verses respective concentration was plotted. [14]

Analysis of marketed formulation

A quantity of the cream equivalent to 80 mg Fluconazole and 1 mg Beclomethasone dipropionate was transferred to a100 ml Volumetric flask, add 60 ml Mobile phase heat on water bath at 60^{o}C temperature for 15 minutes and made up volume up to the mark with mobile phase. The solution was filtered through 0.22 µm Nylon filter and first few drop of filtrate were discarded. 1 ml of this solution was diluted to 10 ml with mobile phase. The solution was injected 20 µl. The areas of resulting peak were measured at 220 nm.

Force degradation study [15]

Acid degradation

1 ml of stock solution and 2 ml of 0.1 N HCl solutions were added into 10 ml of volumetric flask. This mixture was refluxed for 3 h at room temperature. The resultant solution was diluted with mobile phase to obtain Fluconazole (80µg/ml) and Beclomethasone dipropionate (1µg/ml) and chromatogram was recorded (Figure 11).

Base degradation

1 ml of stock solution and 2 ml of 0.1 N NaOH solutions were added into 10 ml of volumetric flask. This mixture was refluxed for 3 h at room temperature. The resultant solution was diluted with mobile phase to obtain Fluconazole (80µg/ml) and Beclomethasone dipropionate (1µg/ml) and chromatogram was recorded (Figure 12).

Oxidation degradation

Oxidation degradation was performed by transferring 1 ml of stock solution to 10 ml of volumetric flask. 2 ml of 3% H_2O_2 solution was added and mixed well and kept for 3 hrs at room temperature. Then the volume was adjusted with diluent to get Fluconazole (80 µg/ml) and Beclomethasone dipropionate (1 µg/ml) and chromatogram was recorded (Figure 13).

Photo degradation

Photo degradation was performed by transferring 1 ml of stock solution to 10 ml of volumetric flask. The flask was kept in UV Chamber for 12 hrs. Then the volume was adjusted with diluent to get Fluconazole (80 µg/ml) and Beclomethasone dipropionate (1 µg/ml) and chromatogram was recorded (Figure 14).

Thermal degradation

Thermal degradation was performed by transferring 1 ml of stock solution to 10 ml of volumetric flask. The flask was kept in oven at 800 °C temperature for 12 hrs. Then the volume was adjusted with diluent to get Fluconazole (80 µg/ml) and Beclomethasone dipropionate (1 µg/ml) and chromatogram was recorded (Figure 15).

3. Results and Discussion

The main objective of this study was to develop a new Stability indicating RP – HPLC method, for simultaneous analysis of Beclomethasone dipropionate and Fluconazole in pharmaceutical dosage form and validate it as per ICH guidelines. [14, 16]

Selection of wavelength

By appropriate dilution of two standard drug solutions with Mobile phase, solutions containing 80 µg/ml of Fluconazole and 1 µg/ml of Beclomethasone dipropionate were scanned in the range of 200 – 400 nm. The overlay spectra of both drugs were recorded (Figure 3). From overlay spectra maximum wavelength selected for detection was 220 nm.

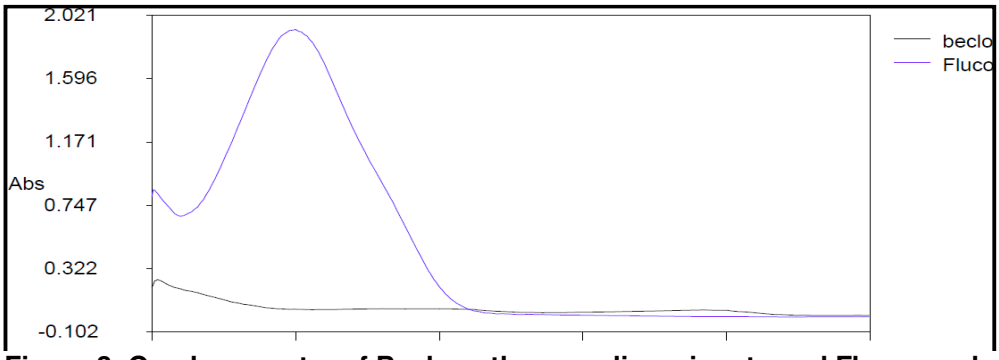


Figure 3. Overlay spectra of Beclomethasone dipropionate and Fluconazole

Method Development

In order to develop stability indicating RP-HPLC method, preliminary study for the analysis of the drugs in terms of parameters like detection wavelength, suitable mobile phase selection and optimum pH was carried out. The method was optimized to get good peak, resolution and other parameters. It was initially tried with different mobile phases like Water: Methanol, water: Acetonitrile, buffer: Methanol, buffer: Acetonitrile in different concentrations and finally the mobile was optimized based on separation efficiency achieved with phosphate buffer 0.05 M (pH 4.0): Methanol (65:35%v/v), pumped at the flow rate1.0 ml/min at 26^{o}C. For estimation of Beclomethasone dipropionate and Fluconazole in formulations, a BDS Hypersil C18 (250mm× 4.6mm, 5µ) was used. Different flow rates (0.5, 0.8, 1.0 and 1.2) were examined and observed that 1.0 ml/min was good one because of better visibility and resolution of the peaks. Ultraviolet detection at 220 nm was utilized because it was discovered to be optimum wavelength for Beclomethasone dipropionate and Fluconazole. Retention time of Beclomethasone dipropionate and Fluconazole was found to be 7.407min min and 4.067 min. using these conditions, good separation of Beclomethasone dipropionate and Fluconazole with acceptable peak shape, resolution and sensitivity was shown in Figure 4. [12, 13, 16]

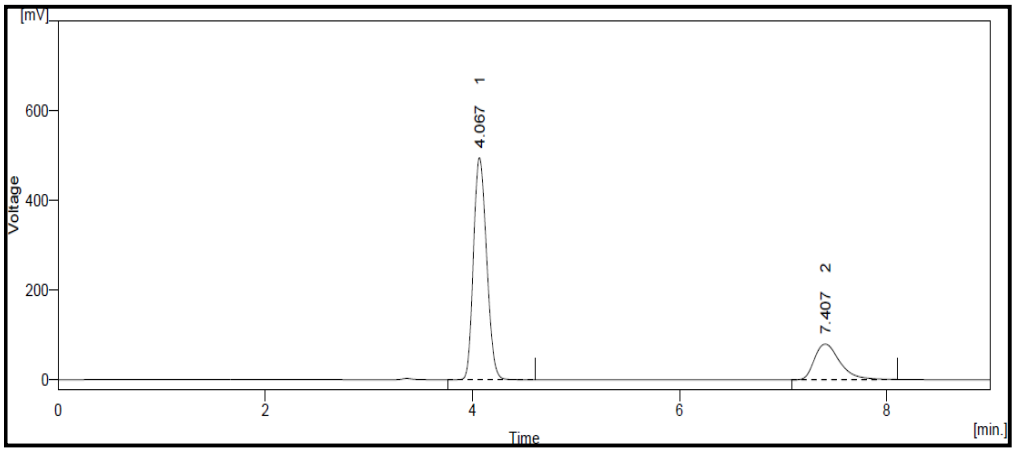


Figure 4. Optimized Chromatogram of Standard Solution of Fluconazole and Beclomethasone Dipropionate in Phosphate Buffer (Ph 4.0): Methanol (65:35 %V/V)

Method Validation

Linearity

The linearity for Fluconazole and Beclomethasone dipropionate were assessed by analysis of combined standard solution in range of 40-120µg/ml and 0.5-1.5µg/ml respectively. Correlation co-efficient for calibration curve Fluconazole and Beclomethasone dipropionate was found to be 0.999 and 0.999 respectively. The visual characteristics such as linearity range, slope, intercept, correlation coefficient and regression linear equation are shown in Figure 5.

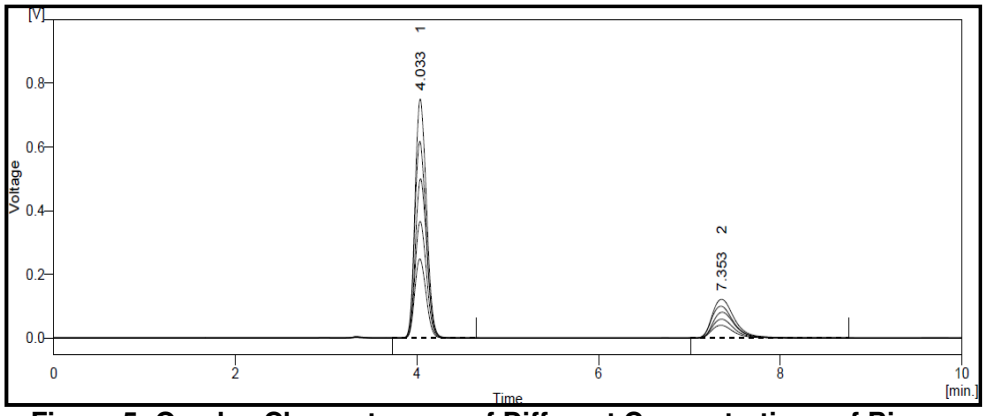


Figure 5: Overlay Chromatogram of Different Concentrations of Binary Mixtures of Fluconazole and Beclomethasone Dipropionate

Table 1. Linearity Data for Fluconazole and Beclomethasone Dipropionate

Fluconazole		Beclomethasone dipropionate	
Concentration (µg/ml)	Area (n=3)	Concentration (µg/ml)	Area (n=3)
40	2171.289	0.5	682.424
60	3205.757	0.75	1015.305
80	4383.295	1.0	1388.885
100	5397.235	1.25	1710.713
120	6567.476	1.5	2081.916

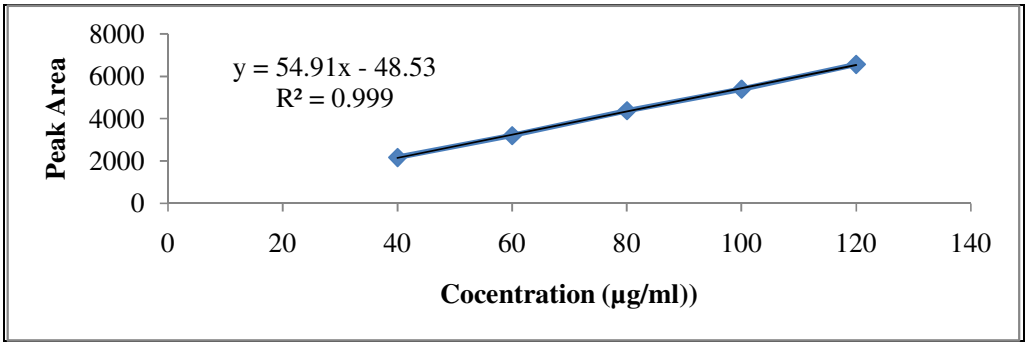


Figure 6. Calibration Curve of Fluconazole

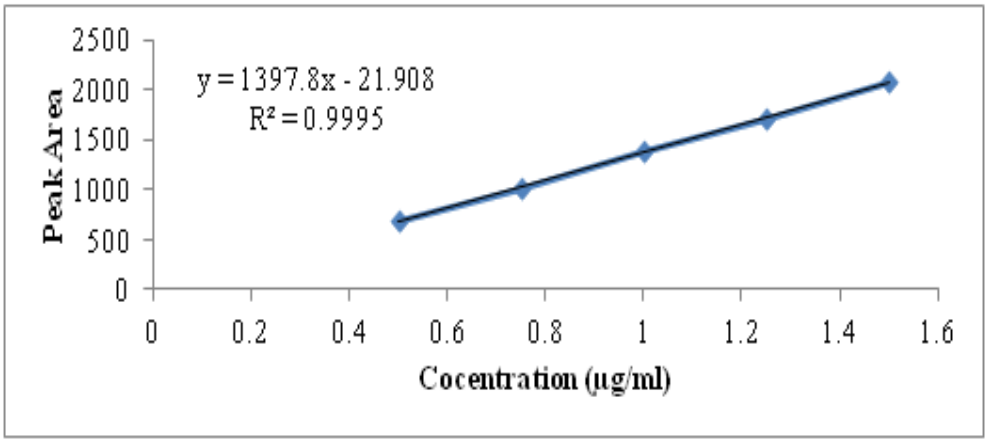


Figure 7. Calibration Curve of Beclomethasone dipropionate

Precision

Method precision (Repeatability)
Repeatability was studied by calculating the RSD for six measurements of same solution of Fluconazole and Beclomethasone dipropionate, respectively performed on the same day and under same experimental conditions. The corresponding results are shown in Table 7. The %RSD was found to be less than 1.0%.

Intermediate precision (Reproducibility)
Intraday and inter day variations were determined by injecting standard solutions (40-120 µg/ml) of Fluconazole and (0.5-1.5µg/ml) of Beclomethasone dipropionate in triplicate within same day and three different days over a period of week. The corresponding results are recorded in Table 7 and %RSD was found to be less than 2.0%.

Accuracy
The accuracy of the method was study by standard addition method. Accuracy is expressed as % recovery of the standard spiked to previously analyzed sample of dosage form. Known amounts of standard solutions of Fluconazole and Beclomethasone dipropionate were added at 80 %, 100 % and 120 % levels to pre quantified sample solutions of Fluconazole (40 µg/ml) and Beclomethasone dipropionate (0.5 µg/ml). The amounts of Fluconazole and Beclomethasone dipropionate were calculated by applying obtained values to the regression equation of the calibration curve as shown in Table 2 and 3.

Table 2. Recovery studies data of Fluconazole

%Level of recovery	Amount of sample taken (µg/ml)	Amount of standard spiked(µg/ml)	Total amount (µg/ml)	Amount recovered (µg/ml)	Peak area (n=3)	% Recovery
80 %	40	32	72	31.598	3892.770	98.745
	40	32	72	32.233	3913.322	100.728
	40	32	72	32.062	3927.711	100.195
100 %	40	40	80	39.619	4334.353	99.047
	40	40	80	40.058	4358.510	100.144
	40	40	80	39.860	4347.616	99.649
120 %	40	48	88	48.003	4795.961	100.006
	40	48	88	47.608	4774.240	99.184
	40	48	88	47.856	4787.893	99.701

Table 3. Recovery studies data of Beclomethasone dipropionate

%level of recovery	Amount of sample taken (µg/ml)	Amount of standard spiked (µg/ml)	Total amount (µg/ml)	Peak area (n=3)	Amount recovered (µg/ml)	% Recovery
80 %	0.5	0.4	0.9	0.400	1298.166	99.894
	0.5	0.4	0.9	0.397	1306.798	99.290
	0.5	0.4	0.9	0.405	1294.611	101.361
100 %	0.5	0.5	1.0	0.501	1451.696	100.182
	0.5	0.5	1.0	0.507	1456.853	101.493
	0.5	0.5	1.0	0.504	1447.212	100.792
120%	0.5	0.6	1.1	0.608	1604.435	101.301
	0.5	0.6	1.1	0.602	1595.665	100.307
	0.5	0.6	1.1	0.605	1600.309	100.833

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

LOD and LOQ for both drugs were calculated theoretically using following equation as per ICH guidelines. These data show that the method is sensitive (Table 7).
LOD= 3.3 * SD/slope of calibration curve and LOQ=10 * SD/slope of calibration curve
Where, SD = Standard deviation of intercepts

Robustness

Influence of small changes in chromatographic conditions such as flow rate, pH and mobile phase shown in Table 4. A method is said to be robust when the alterations in the study produce no significance changes in the results obtained. The developed method is a robust because no significant changes were observed in recovery percentage and in the retention time of compounds.

Table 4. Robustness Studies of Fluconazole and Beclomethasone dipropionate

Drug	Parameter	Level	Peak area	SD	%RSD
Fluconazole	Flow rate (ml/min)	1.2	4233.902	28.896	0.925
		0.8	4266.092	24.742	0.705
	pH	4.2	2582.925	25.749	0.610
		3.8	2773.536	25.270	0.911
	Mobile phase Ratio (%v/v)	52:48	2634.698	24.552	0.932
		48:52	2767.797	24.742	0.758
Beclomethasone dipropionate	Flow rate	1.2	604.639	6.180	1.022
		0.8	641.273	6.586	1.027
	pH	4.2	590.733	9.714	1.644
		3.8	635.082	7.147	1.125
	Mobile phase Ratio (%v/v)	67:33	602.352	8.350	1.386
		63:37	634.918	5.799	0.913

Specificity

Specificity was performed to exclude the possibilities of interference with excipients in the region of elution of Fluconazole and Beclomethasone dipropionate. The specificity of the method was tested under normal conditions and the result of the tests proved that the

components other than the drug did not produce a detectable signal at retention place of Fluconazole and Beclomethasone dipropionate as shown in Figure 8, 9, 10.

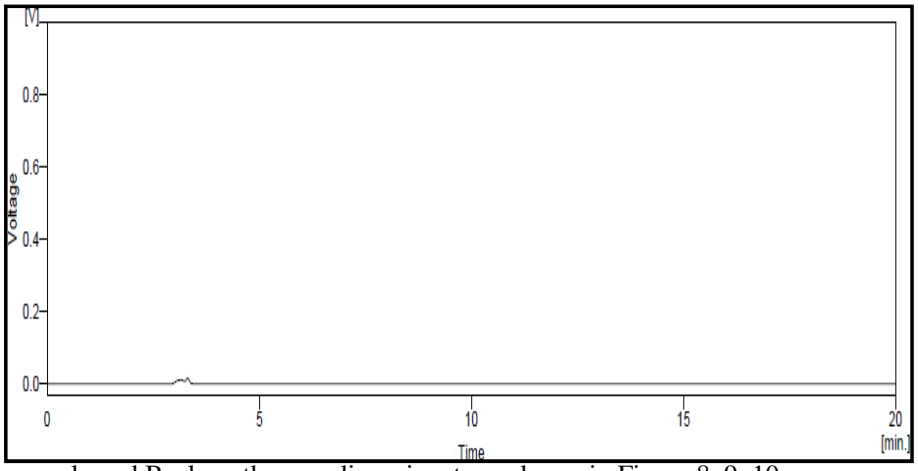


Figure 8. Chromatogram of Blank

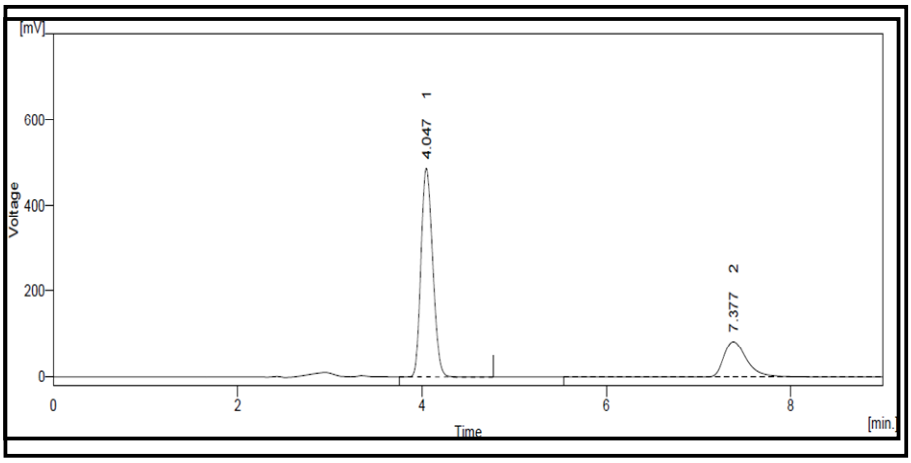


Figure 9. Chromatogram of Sample Fluconazole and Beclomethasone Dipropionate

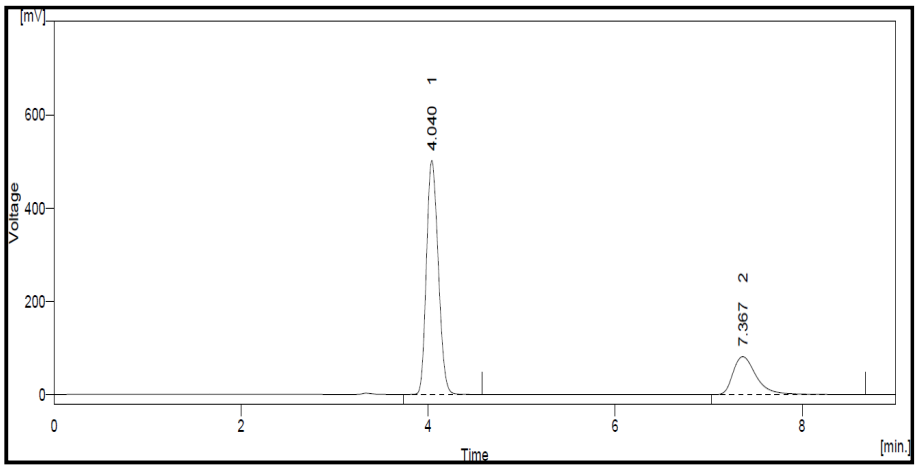


Figure 10. Chromatogram of Standard Fluconazole and Beclomethasone dipropionate

Analysis of marketed formulation by developed method

Applicability of the proposed Method was tested by analyzing the commercially available Cream formulation Fulor-B. Results are shown in Table 5.

Table 5. Analysis of Marketed Formulation

Tablet	Composition		Assay (% of label claim*) Mean ± S. D.	
	Fluconazole	Beclomethasone dipropionate	% Fluconazole	%Beclomethasone dipropionate
Fulor-B	(2% w/w)	(0.025% w/w)	96.754 ± 0.555	99.440 ± 1.803

System suitability parameters

The system suitability test was used to verify that the resolution reproducibility of system was adequate for the analysis. Retention time, number of theoretical plates, tailing factor and resolution were calculated for Fluconazole and Beclomethasone dipropionate. Results are shown in Table 6.

Table 6. System Suitability Parameters

Parameter	Fluconazole	Beclomethasone dipropionate
Retention time (Minutes)	4.067	7.407
Theoretical plates (T_P)	4674	4496
Tailing factor (T_f)	1.303	1.614
Resolution (Rs)	9.827	

Table 7. Summary of Validation Parameter

PARAMETRS		RESULTS	
		Fluconazole	BCL
Linearity Range (n=3)		40µg/ml-120µg/ml	0.5µg/ml-1.5µg/ml
Slope		54.919	1397.8
Intercept		48.53	21.908
Regression equation		y = 54.919- 48.53	y =1397.8-21.908
Correlation co-efficient		0.999	0.999
Limit of detection(µg/ml)		2.788 µg/ml	0.033 µg/ml
Limit of quantification(µg/ml)		8.448 µg/ml	0.100 µg/ml
% Recovery		99.613 - 99.889	100.814 - 100.822
Precision (%RSD)	Repeatability	0.647	1.559
	Intra-day(n=3)	0.308 – 0.790	0.315 – 1.721
	Inter-day (n=3)	0.358 – 0.640	1.049 – 1.600
Specificity		Specific	Specific
Robustness		Robust	Robust
Assay		96.754%	99.440%

Force degradation study

Stability testing forms an important part of the process of drug product development. The purpose of stability testing was to provide evidence on how the quality of a drug substance varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables recommendation of storage conditions,

retest periods, and shelf life to be established. Forced degradation studies were carried out according to ICH guidelines. The objective of the study was to find the likely degradation products, which in turn help in the establishment of degradation pathways and the intrinsic stability of drug. In order to check the selectivity of the proposed method, degradation studies were carried out using hydrolysis (acidic and basic), thermolysis, Photolysis and oxidation. Upon heating all the drugs with 0.1 N NaOH and 0.1N HCl, no reduction of drug peaks in chromatograms was observed as compared to a freshly prepared standard chromatogram; almost 100% recoveries of all the drug were obtained as shown in Table 8.

Acid degradation

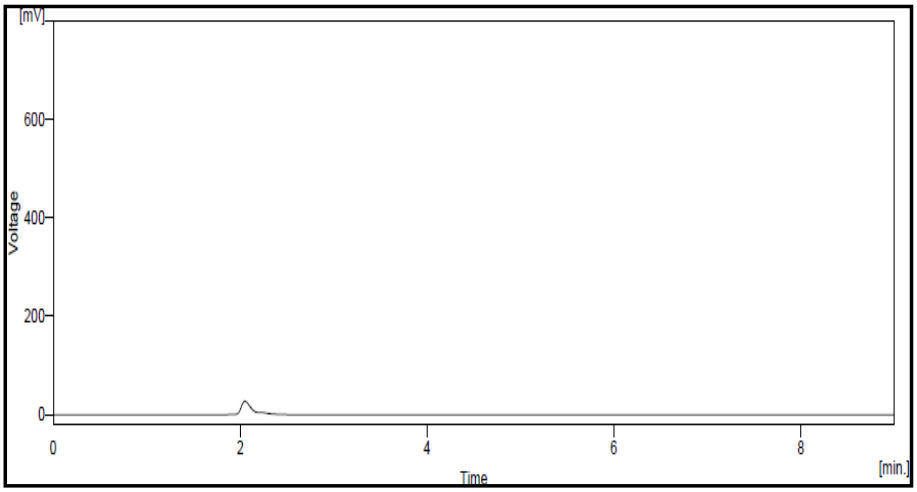


Figure 11. Chromatogram of 0.1 N HCl

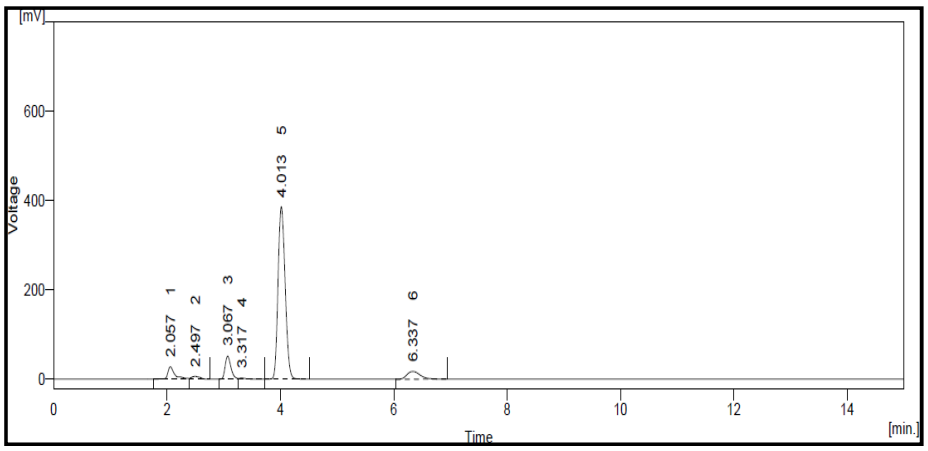


Figure 12 Chromatogram of Standard Fluconazole

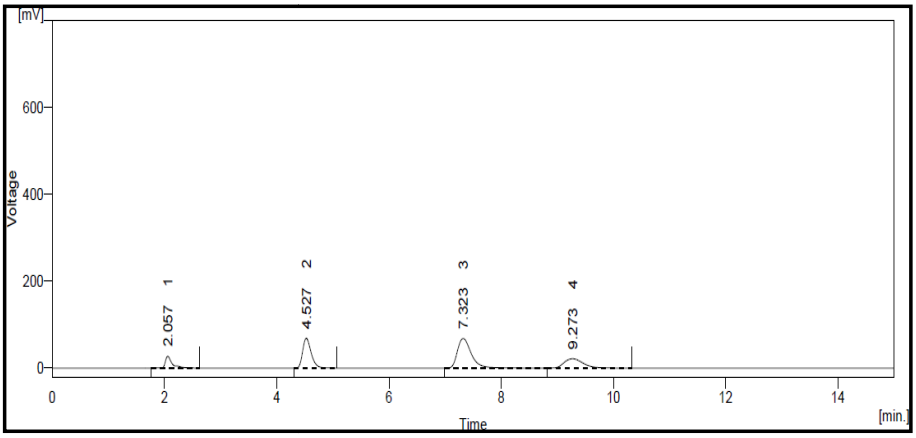


Figure 13. Chromatogram of Standard Beclomethasone Dipropionate

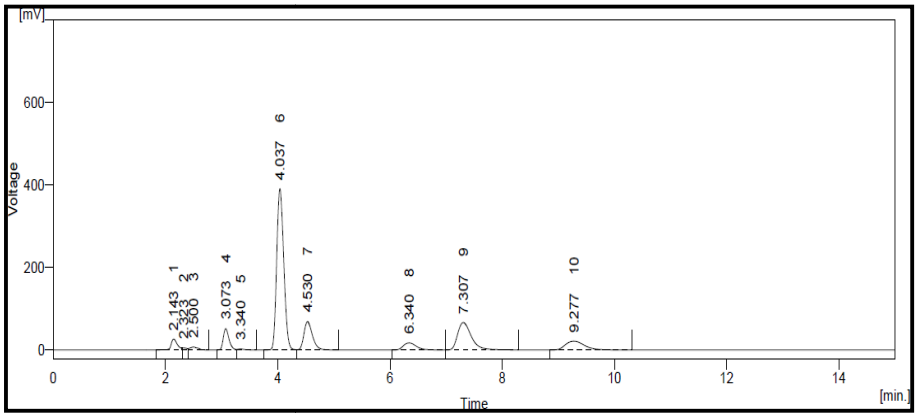


Figure 14. Chromatogram of Sample Mixture of Fluconazole and Beclomethasone Dipropionate

Base Degradation

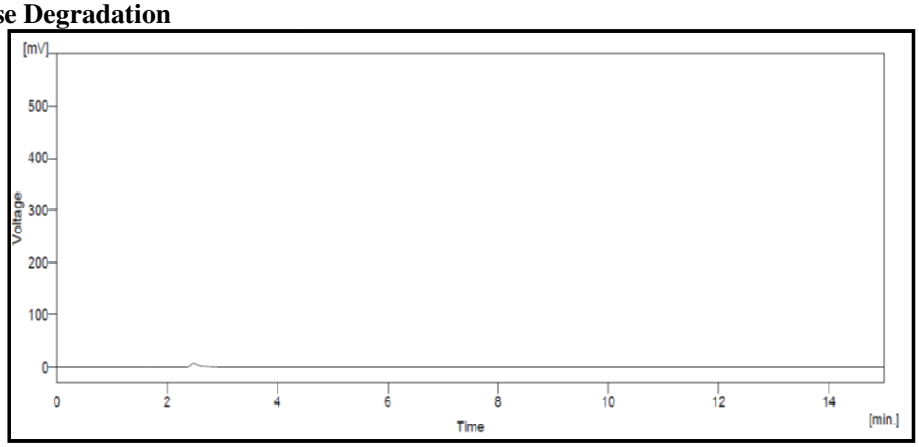


Figure 15. Chromatogram of 0.1 N NaOH

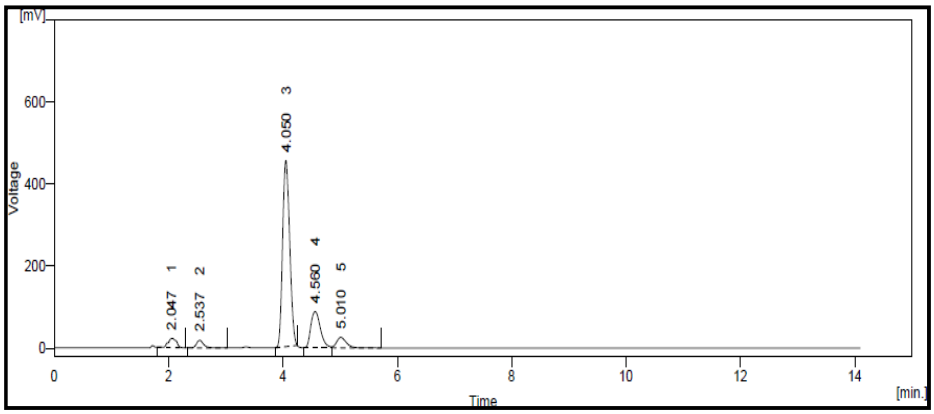


Figure 16. Chromatogram of Standard Fluconazole

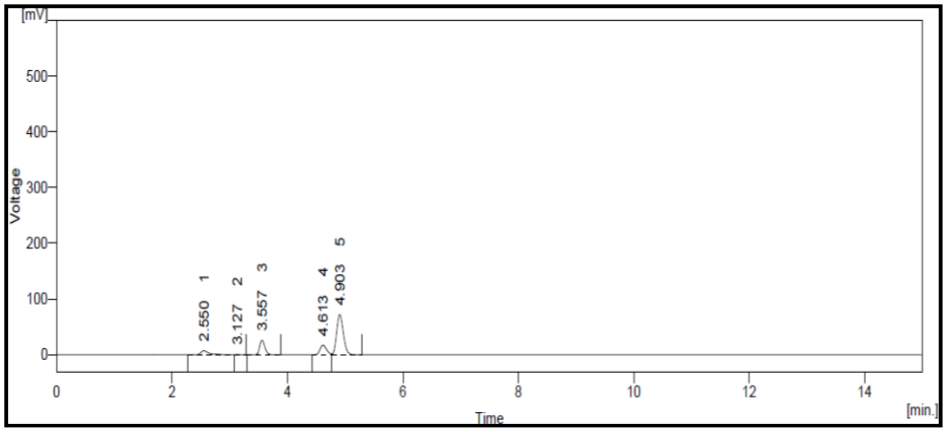


Figure 17. Chromatogram of Standard Beclomethasone Dipropionate

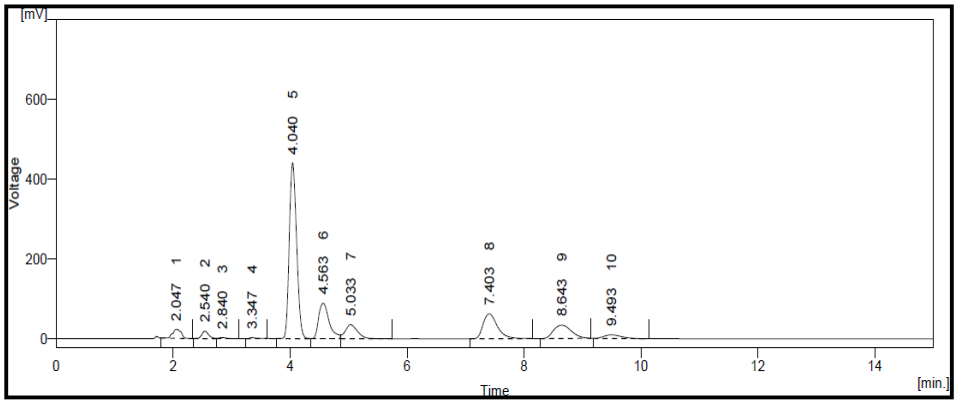


Figure 18. Chromatogram of Sample Mixture of Fluconazole And Beclomethasone Dipropionate

Oxidative degradation

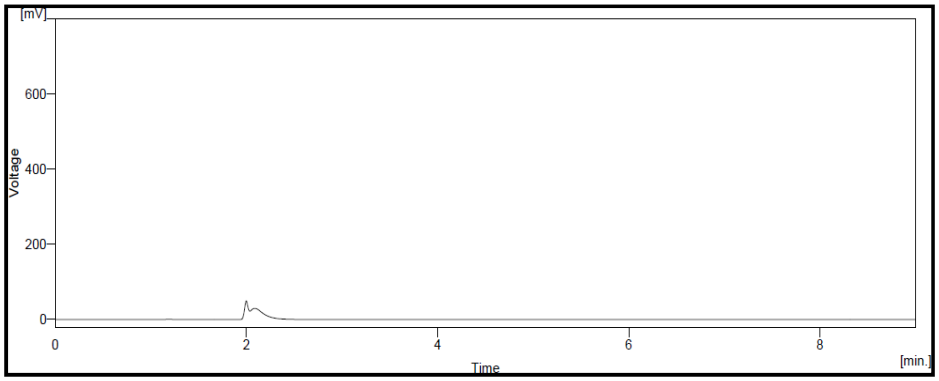


Figure 19. Chromatogram of 3% H_2O_2

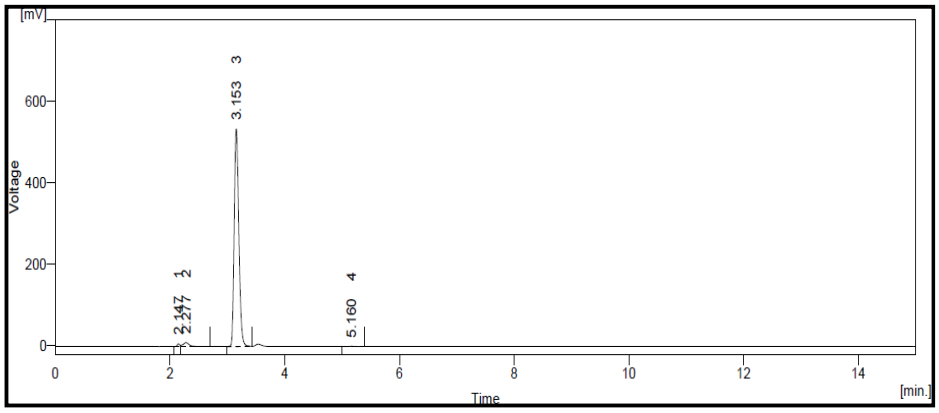


Figure 20. Chromatogram of Standard Fluconazole

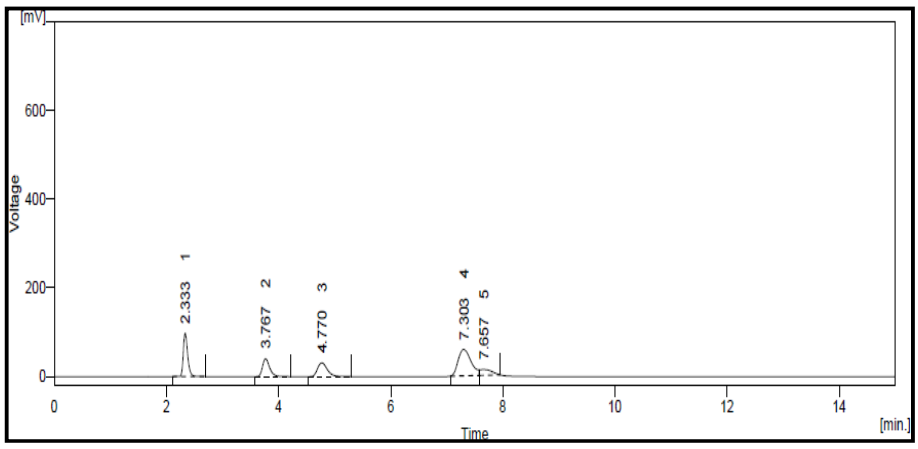


Figure 21. Chromatogram of Standard Beclomethasone Dipropionate

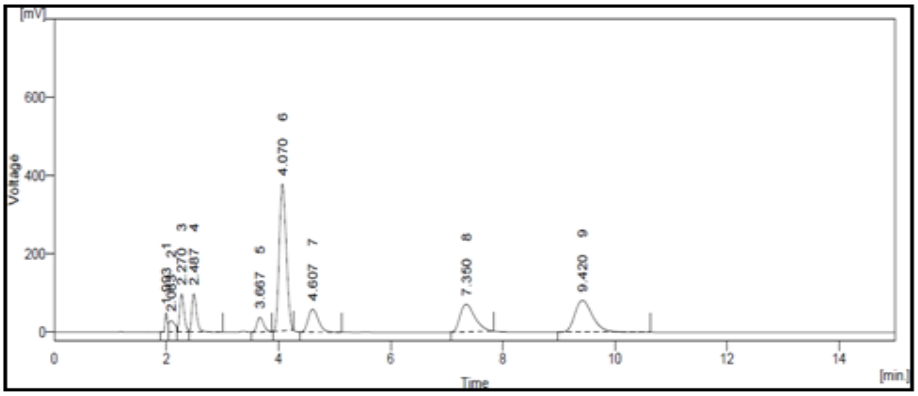


Figure 22. Chromatogram of Sample Mixture of Fluconazole And Beclomethasone Dipropionate

Photo degradation

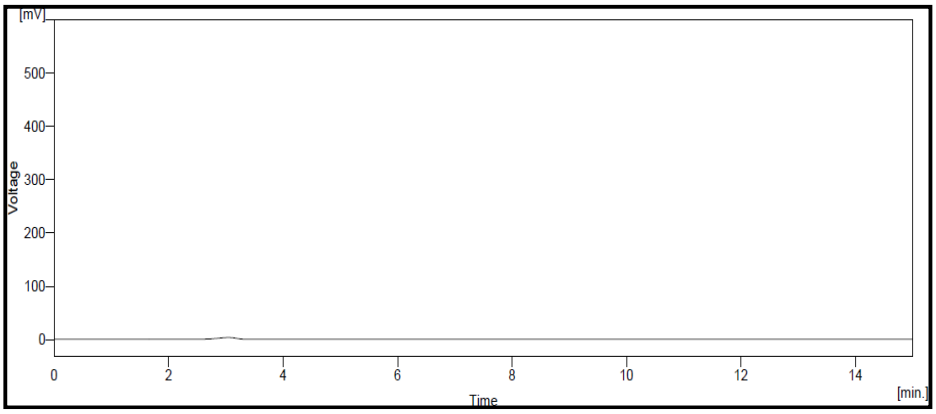


Figure 23. Chromatogram of Photo Degradation Blank

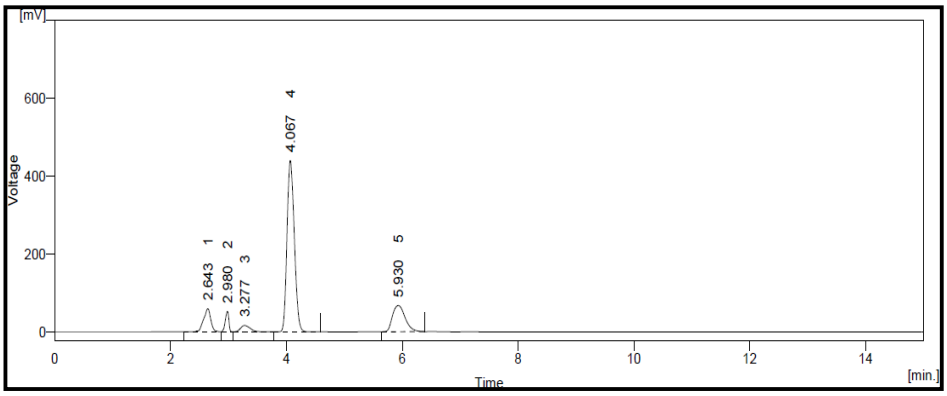


Figure 24. Chromatogram of Standard Fluconazole

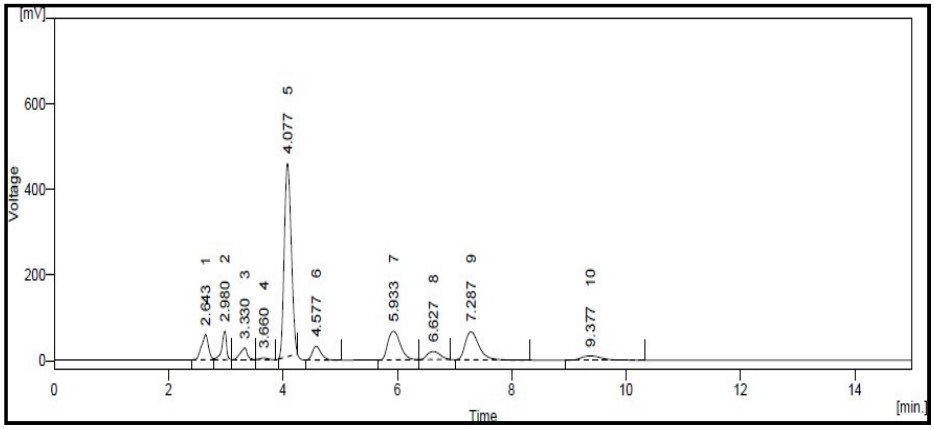


Figure 25. Chromatogram of Standard Beclomethasone Dipropionate

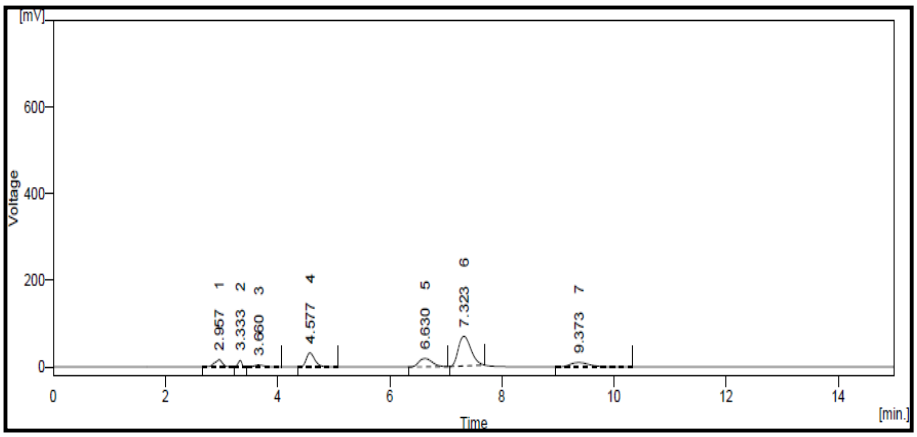


Figure 26. Chromatogram of Sample Mixture of Fluconazole and Beclomethasone Dipropionate

Thermal degradation

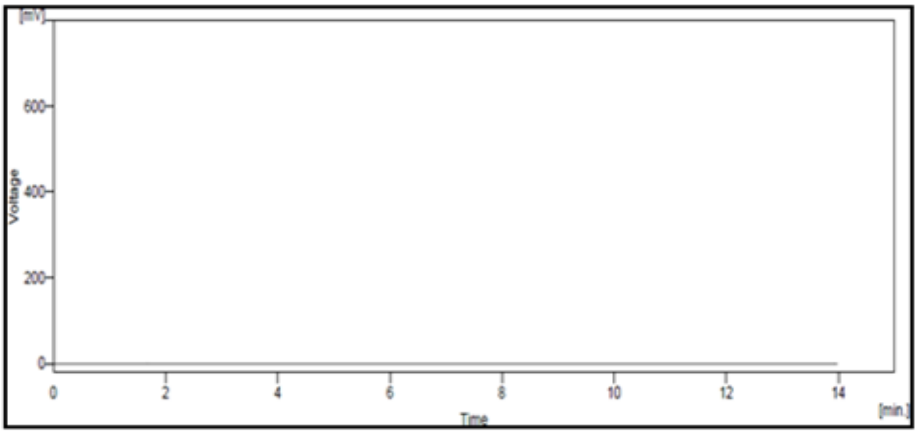


Figure 27. Chromatogram of Thermal Degradation Blank

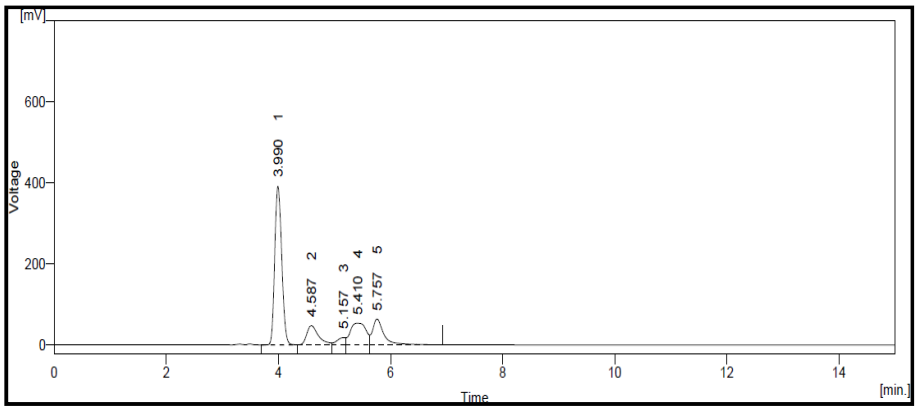


Figure 28. Chromatogram of Standard Fluconazole

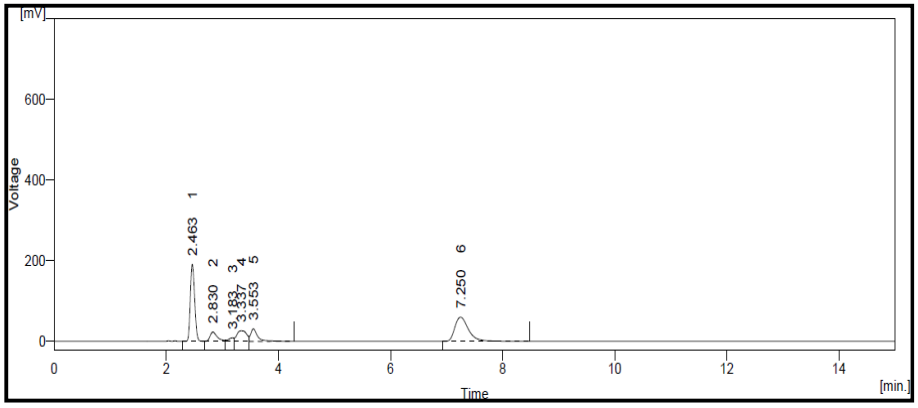


Figure 29. Chromatogram of Standard Beclomethasone Dipropionate

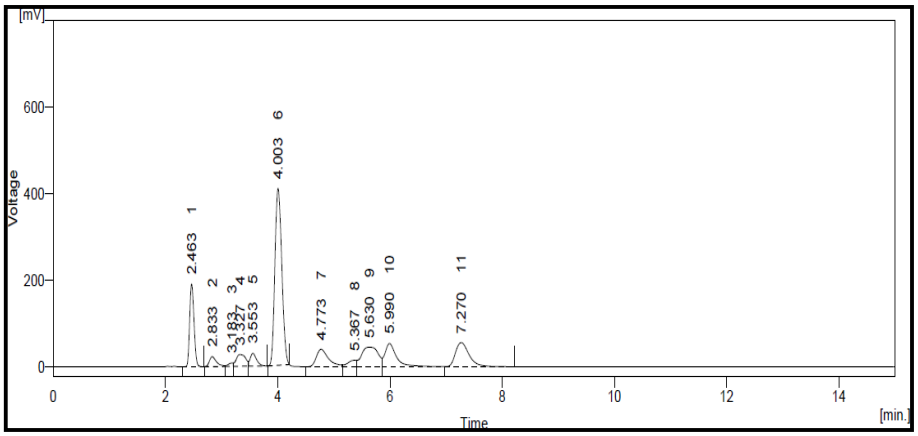


Figure 30. Chromatogram of Sample Mixture of Fluconazole and Beclomethasone Dipropionate

Table 8: Results of Forced Degradation Study

Drug / Parameter	Fluconazole				Beclomethasone dipropionate			
	Standard		Sample		Standard		Sample	
	Area	%Deg.	Area	%Deg	Area	%Deg	Area	%Deg
Acid	3362.11	23.831	3417.67	22.57	1163.48	14.21	1124.62	17.07
Base	3922.18	11.142	3849.93	12.77	1000.27	26.24	1074.30	20.78
Oxidative	2957.27	33.002	3244.51	26.49	949.55	29.98	991.23	26.91
Photo	3877.16	12.162	3868.65	12.35	1075.93	20.66	1104.66	18.55
Thermal	3386.05	23.289	3491.69	20.89	1013.98	25.23	942.47	30.50

%Deg: % Degradation

4. Conclusion

The stability indicating RP-HPLC method for simultaneous estimation of Beclomethasone dipropionate and Fluconazole in pharmaceutical dosage form was developed and validated as per ICH guidelines. Linearity was observed in the range of (0.5-1.5 µg/ml) for Beclomethasone dipropionate and (40-120 µg/ml) for Fluconazole with correlation coefficient ($r^2 = 0.9995$). The % recoveries of Beclomethasone dipropionate and Fluconazole were in the range of 98-102% which was within the acceptance criteria. The %RSD was not more than 2% which proved the precision for the developed method. By studying all the validation parameters (Linearity, Precision, Accuracy, LOD and LOQ) we have concluded that the methods were simple, precise, accurate and rapid for the determination of Beclomethasone dipropionate and Fluconazole in their tablet dosage form. The developed method is a stability indicating that separates degradants and can be conveniently used by quality control department to determine the assay of pharmaceutical preparations and also stability samples. The assay results showed that the method can be successfully applied for routine analysis of Beclomethasone dipropionate and Fluconazole in tablet dosage form.

5. Acknowledgement

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