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Development and Validation of Stability Indicating HPTLC Method for Simultaneous Estimation of Fluocinolone Acetonide and Miconazole Nitrate in Ointment

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Abstract

Fluocinolone acetonide is a steroidal drug and Miconazole nitrate is an antifungal azole. The combination of these drugs has a highly beneficial effect on dermatological inflammatory disorders associated with fungal infections. A simple, specific, accurate, precise, robust and stability indicating HPTLC method has been developed and validated for the simultaneous estimation of Fluocinolone acetonide and Miconazole nitrate in bulk drug and ointment dosage form. Chromatography was performed using pre-coated silica gel aluminium plate $60F_{254}$, (10 ×10 cm) as stationary phase and n-Hexane: Ethyl acetate (1:9, v/v) as mobile phase. Detection was carried out at 254 nm. The R_f value for Miconazole nitrate and Fluocinolone acetonide was found to be 0.46 and 0.64 respectively. The optimized conditions develop showed a linear response from 200-700 ng/spot (r^2 =0.983) for Fluocinolone acetonide and 40000-140000 ng/spot (r^2 =0.990) for Miconazole nitrate. The study involved observation on degradation products formed under different stress condition. The developed method successfully separated drug substances from degradation products formed under various stress conditions.

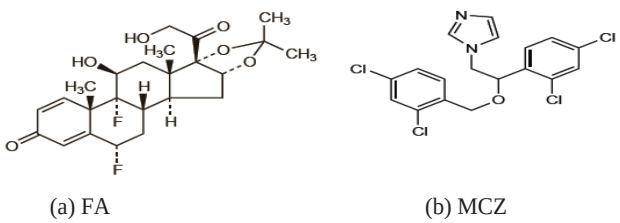
Keywords: Miconazole nitrate; Fluocinolone acetonide; Stability indicating; HPTLC

Introduction

Fluocinolone Acetonide (FA), 6α, 9α-diFAoro-11β, 21-dihydroxy-16α, 17α-isopropylidenedioxy-1, 4-diene, 3, 20-Dione (Figure 1) is a corticosteroid used topically for treatment of a variety of skin disorders and inflammatory eye, ear, and nose diseases [1]. It has high anti-inflammatory activity and is usually used formulated as a cream, gel, lotion, or ointment [2,3]. Miconazole nitrate (MCZ), 1H-imidazole, 1-[2-(2, 4-dichlorophenyl)-2-[(2, 4- icholophenyl) methoxy] ethyl] - (±) nitrate (Figure 1) is an antimycotic imidazole derivative [1]. With a wide activity spectrum, it is endowed with a powerful activity against dermatophytes and Candida albicans, as well as against several Gram positive germens [3,4]. The generally accepted mode of action of azoles antifungal is the inhibition of 14-alpha-lanosterol demethylase, a key enzyme in ergosterol biosynthesis, resulting in depletion of ergo sterol and accumulation of toxic 14-alpha-methylated sterols in the membrane of susceptible yeast species [5]. MCZ has been extensively applied in the management of dermal, oral, and vaginal mycosis. Currently, it is used in a variety of pharmaceutical formulations such as injections, tablets, oral gels, creams, ointments, topical powders and vaginal suppositories. The most usual application forms include creams, ointments or gels at 2.0% concentration level, alone or associated with anti-inflammatory steroids, or other antimicrobials such as gentamycin for the treatment of dermatitis [6,7].

Literature review reveals that detection and quantitative determination of FA and MCZ in routine analysis of individual drug, with combination of other drugs as well as in biological matrices, including human plasma, serum, and urine, is accomplished by variety of methods, including potentiometry [1,8], spectrophotometry [9,10], High Performance Liquid Chromatography (HPLC) [10-21], HPLC with spectrophotometry [14,18], HPLC with Mass Spectroscopy (LC-MS) [22-24], Gas Chromatography (GC) [8,25], Thin Layer Chromatography (TLC) [26], High Performance Thin Layer Chromatography (HPTLC) [27], etc in various dosage forms like cream, gel, tablets, ovule, ointment. However best of our knowledge, no method has been reported yet for simultaneous estimation of above mentioned drugs.

The International Conference on Harmonization (ICH) guideline entitled Stability Testing of New Drug Substances and Products requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [28]. Susceptibility to oxidation is one of the required tests. Also the hydrolytic and the



(a) FA (b) MCZ

Figure 1: Chemical structure of FA (a) and MCZ (b).

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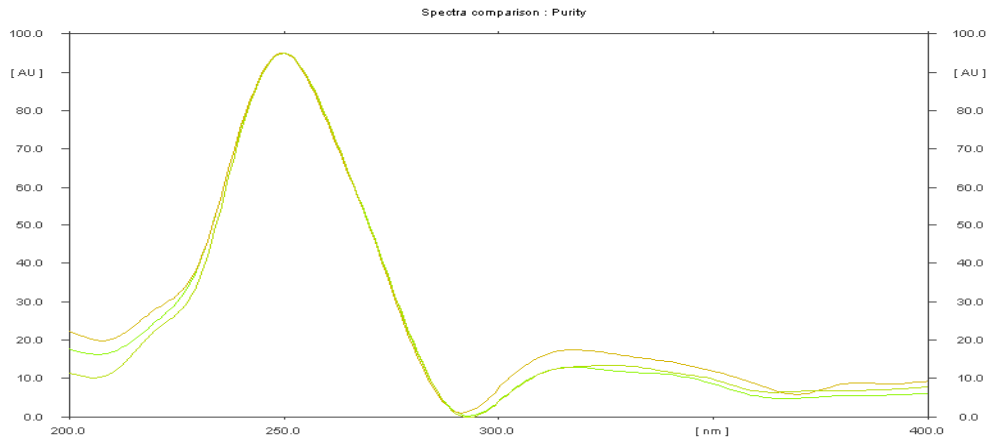

Figure 2: Peak purity spectra of FA in sample.

Table 1: Data of peak purity of sample FA and MCZ.

Substance	r(s, m)	r(m, e)
Fluocinolone acetonide	0.9996	0.9995
Miconazole nitrate	0.9999	1.000

photolytic stability are required. An ideal stability-indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Ferenczi-Fodor and colleagues [29] explained basic acceptance criteria for evaluation of validation experiments based on practical experience for planar chromatographic procedures, which may be used at different levels either in qualitative identity testing, assays, semi-quantitative limit tests or quantitative determination of impurities. The parameters for robustness testing of the given procedures and quality assurance of quantitative planar chromatographic testing have been described as per ICH guidelines.

Nowadays HPTLC is becoming a routine analytical technique due to its advantages of low operating cost, high sample throughput and need for minimum sample clean up. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering the analysis time and cost per analysis.

The aim of the present work was to develop and validate a new simple, rapid, selective, cost effective and stability indicating HPTLC method for simultaneous determination of FA and MCZ in ointment formulation in the presence of its degradation products. The proposed method was validated as per ICH guidelines [30,31].

Experimental

Materials

Pharmaceutical grade of MCZ (Luxica Pharma Inn., Ankleshwer, Gujarat, India) and FA (Dermocare Labs Ltd., Ahmedabad, Gujarat, India) were kindly supplied as a gift sample. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

HPTLC instrumentation

The samples were spotted in the form of bands of width 6mm with a Camag 100 µL syringe on pre-coated silica gelaluminium Plate 60 F–254 (20 × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by An chrome Technologists, Mumbai, India) using a Camag Linomat V(Muttenz, Switzerland). The plates were prewashed by methanol and activated at 60 °C for

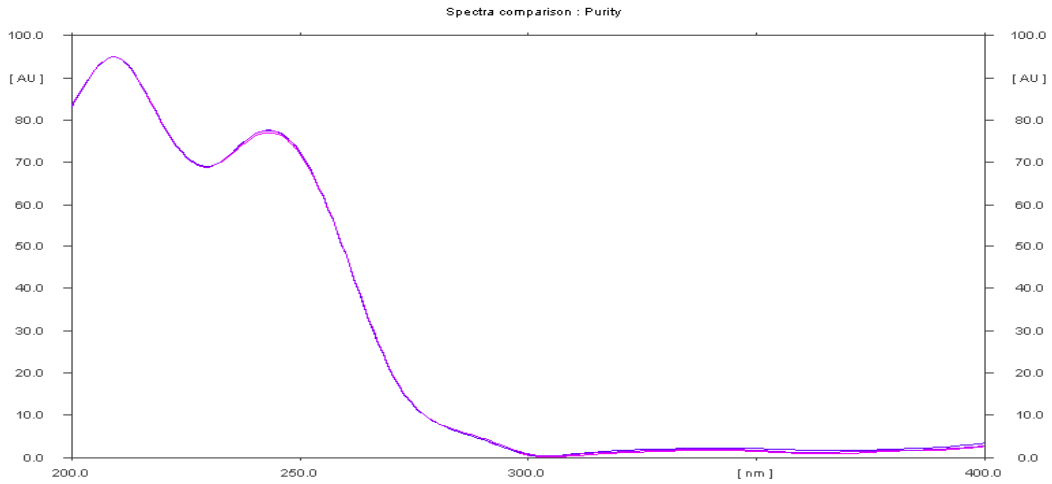

Figure 3: Peak purity spectra of MCZ in sample.

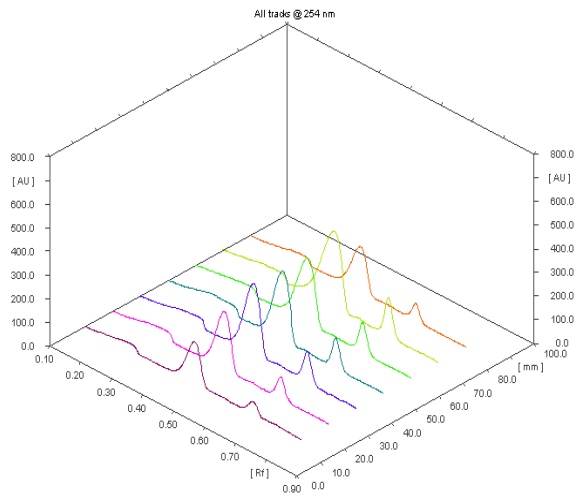

Figure 4: 3D chromatogram of MCZ (40000-140000 ng/spot) and FA (200-700 ng/spot).

Table 2: Calibration data of MCZ.

Sr. No.	Concentration (ng/spot)	Peak Area ± S.D. (n= 6)	%RSD
1	40000	7485.3±93.2	1.24
2	60000	10019.4±91.2	0.91
3	80000	12737.1±94.9	0.74
4	100000	14562±271.07	1.86
5	120000	16625.3±266.86	1.60
6	140000	20387±259.83	1.27

Table 3: Calibration data of FA.

Sr. No.	Concentration (ng/spot)	Peak Area ± S.D. (n= 6)	%RSD
1	200	1466.9±26.55	1.81
2	300	2191.6±10.95	0.49
3	400	2701.6±21.54	0.79
4	500	3202.3±32.50	1.01
5	600	3441.4±32.27	0.93
6	700	4161.7±22.30	0.53

5 min prior to chromatography. A constant application rate of 0.1 µl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 5 × 0.45 mm and scanning speed was employed 10 mm/s. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of n-Hexane/Ethyl acetate (1:9 v/v) and 10 ml of mobile phase was used as per chromatography. Linear ascending development was carried out in 10 × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 °C ± 2) at relative humidity of 60 ± 5%. The length of chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air dryer. Densitometry scanning was performed on Camag TLC scanner III in there flectance–absorbance mode at 260 nm and was operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound

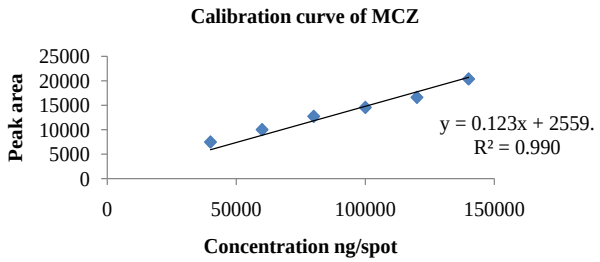

Figure 5: Calibration curve of MCZ at 254 nm.

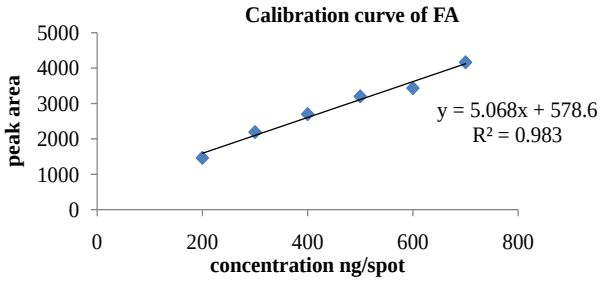

Figure 6: Calibration curve of FA at 254 nm.

chromatographer were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

Preparation of standard solutions

Accurately weighed portion of MCZ (250 mg) and FA (25 mg) was transferred to a separate 25 ml volumetric flask and dissolved and diluted to the mark with methanol to obtain standard solution having concentrations of MCZ (10000µg/ml) and FA (1000µg/ml). Further dilute FA 10 times to obtain standard stock solution having concentration 100 µg/ml.

Preparation of sample solutions

Weigh an accurately about 2 grams of ointment containing 2% of MCZ and 0.01% of FA in 10 ml volumetric flask. Add 2/3rd quantity of methanol into flask and sonicate the solution for 10 min to get clear solution. Add methanol up to mark to make the volume 10 ml. get the test solution contains 4000µg/ml MCZ and 20 µg/ml FA.

Preparation of calibration curve

To set up the linearity range 40000-140000 ng/spot for MCZ and

Table 4: Repeatability data for FA and MCZ.

Sr.No.	Peak Area	
	MCZ(60000 ng/spot)	FA(300 ng/spot)
1	9828.4	2114.7
2	10069.7	2126.7
3	10019.8	2123.1
4	9904.9	2107.5
5	10184.6	2102.4
6	10143.2	2195
Mean	10025.19	2128.23
S.D.	137.51	33.96
% RSD	1.37	1.59

Table 5: Intermediate precision data for MCZ.

Concentration of MCZ (ng/spot)	Intra-day precision		Inter-day precision	
	Area Mean ± SD (n= 3)	%RSD	Area Mean ± SD (n= 3)	%RSD
60000	10019.4 ± 91.20	0.9102	10453.67 ± 146.79	1.40
100000	14562.267 ± 271.07	1.86	14453.67 ± 146.791	1.0155
140000	20387 ± 270.687	1.31	20953.67 ± 322.108	1.548
Mean RSD		1.36	Mean RSD	1.32

Table 6: Intermediate precision data for FA.

Concentration of FA (ng/spot)	Intra-day precision		Inter-day precision	
	Area Mean ± SD (n= 3)	%RSD	Area Mean ± SD (n= 3)	%RSD
300	2191.6 ± 10.95	0.50	2191.6 ± 10.95	0.85
500	3202.3 ± 32.50	1.01	3202.3 ± 32.50	1.60
700	4161.7 ± 22.3	0.53	4161.7 ± 22.3	1.65
Mean RSD		0.68%	Mean RSD	1.36%

200-700 ng/spot FA. From the combined working standard solution (20 µg/ml of FA and 4000 µg/ml of MCZ) aliquots of 10, 15, 20, 25, 30 and 35 µl were spotted on the TLC plate and developed and analysed. The above working standard of MCZ and FA were spotted in band width 6 mm using Hamilton 100 µL syringe on pre-coated silica gel aluminium plate 60 F_{254}using automatic application device. Linear ascending development was carried out in 10 × 10 cm twin trough glass chamber saturated with the mobile phase for 30 min. The plate was removed from the chamber, subsequently dried in a current of air and densitometry scanning was performed on Camag TLC scanner III in the reflectance-absorption mode at 254 nm and operated by win CATS software. Peak areas were recorded for all the peaks. The calibration curve for MCZ and FA were constructed by plotting peak area versus concentration (ng/spot) corresponding to each spot.

Method validation

Precision

a. Repeatability

System precision experiment was performed by application of 15 µL of combined working standard solution (20 µg/mL of FA and 4000 µg/mL of MCZ) for six times on same TLC plate. Plate was developed and analyzed as described in section 4.2.1. The areas of six replicate spots were measured and % RSD was calculated.

b. Intermediate Precision (Reproducibility)

The intra-day and inter-day precision of the proposed method was determined by analyzing mixed standard solution of FA and MCZ at 3 different concentrations (200, 400, 600 ng/spot for FA and 40000, 80000, 120000 ng/spot for MCZ) on the same day and on different days. The results are reported in terms of relative standard deviation (%RSD).

Robustness of the method: By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like n- Hexane/ Ethyl acetate (0.9:9.1 v/v) and (1.1:8.9, v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ± 5%. The plates were prewashed by methanol and activated at 60 ± 5°C for 2, 5 and 7min. prior to chromatography. Time from spotting to chromatography and from chromatography to scanning varied from 0, 25 and 35 min. Robustness of the method was done at 4000 ng/spot of MCZ and 20 ng/spot FA.

Limit of detection and limit of quantization: The limits of detection and quantification of the developed method were calculated from the standard deviation of the intercepts and mean slope of the calibration curves of FA and MCZ using the formulae as given below.

LOD = 3.3 Xσ/S

LOQ = 10 Xσ/S

Where, σ = the standard deviation of the response

S = slope of the calibration curve.

Specificity: The specificity of the method was as certained by analysing standard drug and sample. The spot for MCZ and FA in sample was confirmed by comparing the R_f and spectra of the spot with that of standard. The peak purity of MCZ and FA were assessed by comparing the spectra at three different levels; i.e. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Accuracy: The samples were spiked with extra 80%, 100%and 120% of the standard MCZ and FA solutions, and the mixtures were reanalysed by the proposed method. The experiment was conducted

Table 7: Recovery data of FA from formulation.

Sr. No.	Amount taken (ng/spot)	Amount added (ng/spot)	Total (ng/spot)	Area	Amount Recover (ng/spot)	% Recovery	Mean % Recovery
1	300	240	540	3320.6	241.04	100.43	100.63
		240	540	3293.6	235.71	98.21	
		240	540	3354.9	247.81	103.25	
2	300	300	600	3621.4	300.39	100.13	99.44
		300	600	3615.7	299.27	99.75	
		300	600	3595.6	295.30	98.43	
3	300	360	660	3933.4	361.95	100.54	100.41
		360	660	3914.6	358.24	99.51	
		360	660	3945.2	364.28	101.19	
Mean Recovery = 100.16%							
Standard Deviation = 0.63							
% Relative Standard Deviation = 0.63%							

Table 8: Recovery data of MCZ from formulation.

Sr No.	Amount taken (ng/spot)	Amount added (ng/spot)	Total (ng/spot)	Area	Amount Recover (ng/spot)	% Recovery	Mean % Recovery
1	60000	48000	108000	15956.4	48918.71	101.91	99.59
		48000	108000	15865.4	48182.13	100.38	
		48000	108000	15635.7	46314.65	96.49	
2	60000	60000	120000	17213.6	59143.14	98.57	98.73
		60000	120000	17358.4	60320.36	100.53	
		60000	120000	17105.9	58267.51	97.11	
3	60000	72000	132000	18667.4	70962.65	98.55	97.88
		72000	132000	18420.9	68958.53	95.77	
		72000	132000	18735.1	71513.11	99.32	
Mean Recovery = 98.73%							
Standard Deviation = 0.85							
% Relative Standard Deviation = 0.86%							

Table 9: LOD and LOQ data for FA and MCZ nitrate.

Parameters	FA	MCZ
Standard deviation of the Y- intercepts of the three calibration curves.	17.71	107.33
Mean slope of the three calibration curves.	5.06	0.12
LOD = 3.3 × (SD/Slope) (ng/spot)	11.54	2887.56
LOQ = 10 × (SD/Slope) (ng/spot)	34.97	8750.20

Table 10: Robustness parameters for FA and MCZ.

Sr. No.	FA			MCZ
Change in saturation time				
	25 min	**35 min**		**25 min**
1	2114.7	2123.1	1	2114.7
2	2126.7	2107.5	2	2126.7
Mean	2118			9955.7
S.D	8.61			109.38
%RSD	0.40			1.09
Chang in Mobile phase ratio: n- Hexane: Ethyl acetate				
	0.9:9.1, v/v	**1.1:8.9, v/v**		**0.9:9.1, v/v**
1	2058.28	2060.63	1	2058.28
2	2112.51	2131.08	2	2112.51
Mean	2102.6			9962.63
S.D	26.06			89.92
% RSD	1.23			0.90
Change in sonication time for sample preparation:				
	5 min	**15 min**		**5 min**
1	2102.7	2113.1	1	2102.7
2	2085.4	2107.5	2	2085.4
Mean	2102.175			10090.62
S.D	11.96			120.17
% RSD	0.56			1.19

in triplicate. This was done to check for the recovery of the drug at different levels in the formulations.

Analysis of the marketed formulation

20 µl of sample solution was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate. The amount MCZ and FA present in the sample solutions were determined by fitting the response into the respective regression line equation for MCZ and FA.

Forced degradation

Forced degradation of FA and MCZ was carried out under acidic and alkaline hydrolysis, oxidative and photolytic stress conditions.

Acidic hydrolysis: Accurately weighed 2 mg of FA and 400 mg of MCZ was transferred into 10 ml volumetric flask, dissolved in 5 ml methanol and diluted up to the mark with 2N HCl. The solution was refluxed for 1 hour at room temperature. Aliquot of 1 ml was transferred to 10 ml volumetric flask and neutralized by 2N NaOH. Volume was made up to mark with methanol. The resulting solution (15 µl) was applied to TLC plate and the chromatogram was developed.

Alkaline hydrolysis: Accurately weighed 2 mg of FA and 400 mg of MCZ was transferred into 10 ml volumetric flask, dissolved in 5 ml methanol and diluted up to the mark with 2N NaOH. The solution was refluxed for 1 hour at room temperature. Aliquot of 1 ml was transferred to 10 ml volumetric flask and neutralized by 2N HCl. Volume was made up to mark with methanol. The resulting solution (15 µl) was applied to TLC plate and the chromatogram was developed.

Oxidative degradation: Accurately weighed 2 mg of FA and 400 mg of MCZ was transferred into 10 ml volumetric flask, dissolved in 5 ml methanol and diluted up to mark with 6% hydrogen peroxide (H_2O_2). The solution was kept at room temperature (25± 2^{0}C) for 1 hour. From sample solution 1 ml aliquot was transferred in to 10 ml volumetric flask and volume made up to mark with methanol. The resulting solution (15 µl) was applied to TLC plate and the chromatogram was developed.

Photolytic degradation: For photolytic degradation studies, working standard solution of FA (20µg/ml) and MCZ (4000µg/ml) was exposed to direct sunlight (afternoon, 32±0.5°C) for 3 hr on a

Table 11: Estimation of FA and MCZ in pharmaceutical formulation (Zole-F ointment).

Drug	Label claim	Amount found	% Content
FA	0.01% w/w	0.009882% w/w	98.82%
MCZ	2.0% w/w	1.9994% w/w	99.97%

Table 12: Summary of validation parameters.

Sr. No.	Parameters	FA	MCZ
1	Linearity Range	200-700 ng/spot	4000-140000 ng/spot
2	Correlation Co-efficient (R^2)	0.990	0.983
3	Precision (% RSD) Repeatability (n=6) Intraday precision (n=3) Inter day precision (n=3)	 1.54 0.681 1.366	 1.76 1.36 1.32
4	% Recovery	100.163%	98.79%
5	Limit of Detection (LOD)	11.5424	2887.56
6	Limit of Quantification (LOQ)	34.9769	8750.20

Table 13: Integration parameters for HPTLC method.

Parameters	Value
Video integration	no
Baseline correction	yes
Peak thresh. height	10 AU
Peak thresh. area	300
Peak thresh. slope	3
Filter factor	3
Data selection factor	1
Track start position	85 mm
Track end position	135 mm

wooden plank kept on terrace. 15 µl of the solution was applied on TLC plate and the chromatogram was developed.

Results and Discussion

Mobile phase optimization

The HPTLC procedure was optimized with a view to develop stability indicating assay method. Both, MCZ and FA were spotted on TLC plates and run in different mobile phase systems. The mixture of n-Hexane: Ethyl acetate (1:9, v /v) was proven to be better than the other mixtures in terms of resolution and peak shape.

Validation of the proposed method

Specificity: The peak purity of FA and MCZ were assessed by comparing its HPTLC chromatogram with standard at peak start, apex, and peak end positions of the spot.

Good correlation (r= 0.9990) was obtained between standard and sample spectra of FA and MCZ respectively.

Peak purity spectra of test FA and MCZ are shown in Figures 2 and 3 respectively. Data of peak purity are shown in Table 1.

Linearity: Linear responses were observed in the concentration range of 200-700 ng/spot for FA and 40000-140000 ng/spot for MCZ. Correlation co-efficient for calibration curve of MCZ and FA were found to be 0.990 and 0.983 respectively. 3D chromatogram of standard MCZ and FA in linearity range is depicted in Figure 4.

The data for linearity range of MCZ and FA is depicted in Table 2 and 3 respectively. Calibration curves of MCZ and FA are shown in Figure 5 and Figure 6 respectively.

The regression line equation for MCZ and FA are as following

$y = 0.123x + 2559$ for MCZ

$y = 5.068x + 578.6$ for FA

Where, y= Peak area

x= Concentration in ng/spot

Precision

System and method precision: %RSD of system precision of FA and MCZ were found to be 1.37 and 1.59 respectively. %RSD of method precision of FA and MCZ were found to be 1.75 and 1.63 respectively. The data for system and method precision of FA and MCZ are depicted in Table 4 and 5 respectively.

Intra-day and Inter-day precision: Mean RSD for intra-day and inter-day precision of MCZ was found to be 1.36 and 1.32 respectively. The Mean RSD for intraday and inter day precision of FA was found to be 0.6818 and 1.36 respectively. The data for intra-day and inter-day precision for MCZ is depicted in Table 6 and for FA in Table 7.

These results indicate that method is precise.

Accuracy: Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The % recovery of FA and MCZ was found to be 100.163 and 98.73 respectively. The data for accuracy of FA and MCZ are depicted in Table 7 and Table 8 respectively. These results indicate that method is accurate.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD for FA and MCZ were found to be 11.71 ng/spot and 2887.56 ng/spot respectively.

The LOQ for FA and MCZ were found to be 34.9769 ng/spot and 8750.20 ng/spot respectively. The data for LOD and LOQ of FA and MCZ are depicted in Table 9.

Robustness: Robustness data clearly shows that the proposed

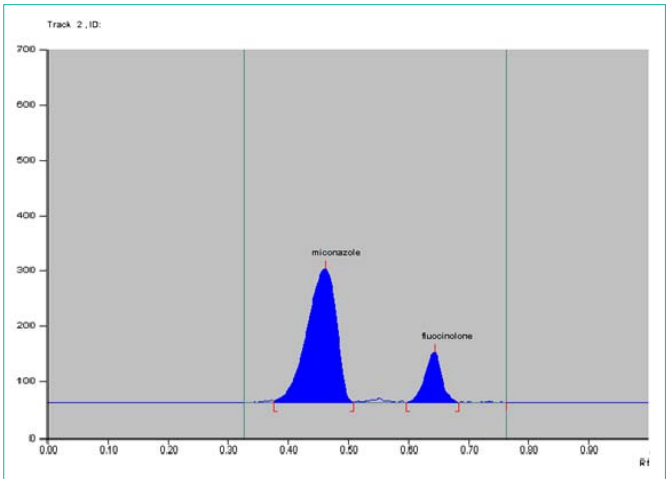

Figure 7: Densitogram of test peak containing 60000 ng/spot MCZ and 300 ng/spot FA.

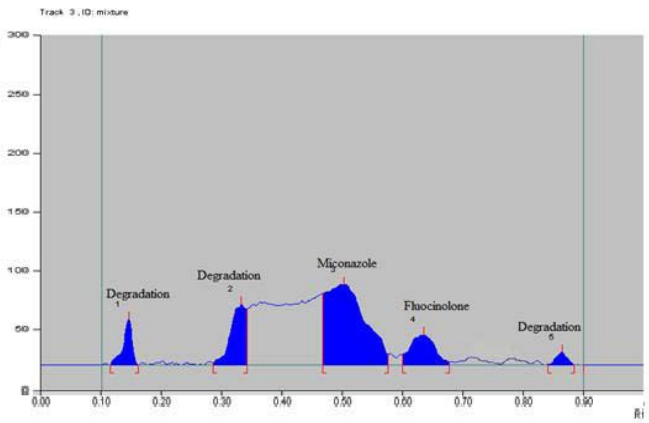

Figure 8: Densitogram of acid hydrolysis.

Table 14: Peak table of acid degradation study.

Peak No.	R_f value	% Area	Substance
1	0.14	10.65	Degradation
2	0.32	14.79	Degradation
3	0.50	49.47	MCZ
4	0.64	19.53	FA
5	0.87	5.26	Degradation

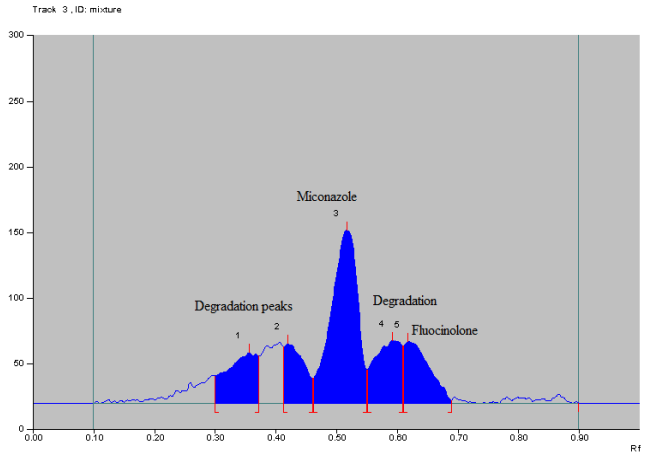

Figure 9: Densitogram of alkaline hydrolysis.

Table 15: Peak table of alkali degradation.

Peak No.	R_f value	% Area	Substance
1	0.36	12.71	Degradation
2	0.42	10.65	Degradation
3	0.51	44.62	MCZ
4	0.59	16.53	Degradation
5	0.64	15.49	FA

method is robust at small but deliberate change. Robustness data are depicted in Table 10 and 11.

Summary of all validation parameters are depicted in Table 12.

Analysis of marketed formulations (ointment) by proposed method

The assay results obtained by using the proposed method for

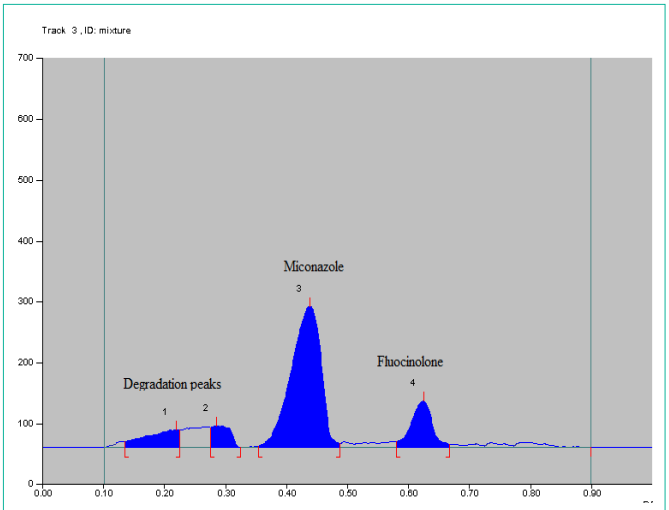

Figure 10: Densitogram of oxidation induced degradation study.

Table 16: Peak table of oxidative degradation.

Peak No.	R_f value	% Area	Substance
1	0.21	5.43	Degradation
2	0.29	10.74	Degradation
3	0.45	62.57	MCZ
4	0.64	20.26	FA

the analysis of a marketed ointment formulation containing FA (0.01%, w/w) and MCZ (2%, w/w) were in good agreement with the labeled amounts of FA and MCZ. The average contents of FA and MCZ were 0.009882g/100g ointment (0.009882% w/w) and 1.9994g/ 100g ointment (1.9994 %, w/w), respectively. So the % drug

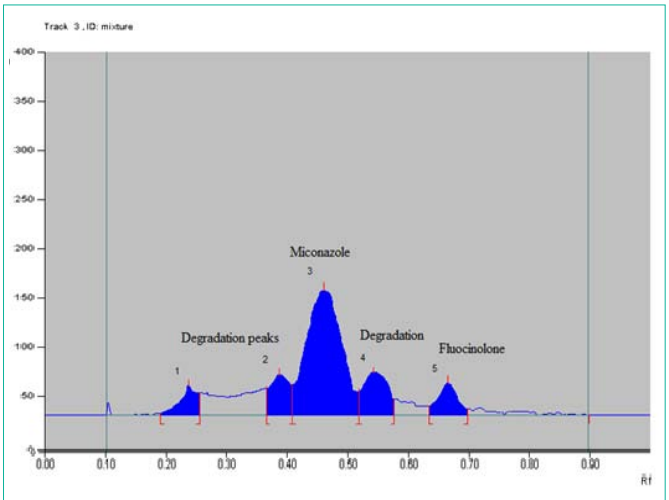

Figure 11: Densitogram of photodegradation.

Table 17: Peak table of Photo degradation.

Peak No.	R_f value	% Area	Substance
1	0.23	8.76	Degradation
2	0.39	9.39	Degradation
3	0.46	47.88	MCZ
4	0.54	17.48	Degradation
5	0.66	16.49	FA

Table 18: Summary of forced degradation study of FA and MCZ.

Sr. No.	Stress type*	Stress condition	Peak area of FA	Peak area of MCZ	% Degradation
1	Acid hydrolysis	1 N HCl at Room temperature, for 1 hour	973.1	4153.8	30.7%
2	Alkali hydrolysis	1 N NaOH at Room temperature, for 1 hour	1837.1	5374	40%
3	Oxidation	3% W/V H_2O_2 at Room temperature, for 1 hour	2430	10514	16.17%
4	Photolytic degradation	Direct sunlight for 3 hour	976.8	12018.9	35.63%

*Concentration of FA: 300 ng/spot and for MCZ: 60000 ng/spot.

obtained was 98.82% of FA and 99.97% of MCZ. No interference of the excipients with the peaks of interest appeared; hence the proposed method is applicable for the routine estimation of FA and MCZ in pharmaceutical dosage forms. Results obtained are shown in following Table 13. Densitogram of test solution containing MCZ (60000 ng/spot) and FA (300 ng/spot) is depicted in Figure 7.

Stability indicating property

Acid hydrolysis: The rate of degradation in acid was found to be slower as compared to that of alkali. After applying 1 N HCl exposure for 1 hour, about 30.7% degradation was observed. Three additional peak of degradation product was observed at R_f 0.12, 0.32 and 0.87 (Figure 8). Data of acid hydrolysis is given in Table 14.

Alkaline hydrolysis: The drug was found to be highly labile to alkaline hydrolysis. After applying 1 N NaOH exposure for 1 hour, about 40% degradation was observed. Additional peak of degradation product were observed at R_f 0.36, 0.42 and 0.59 (Figure 9). Data of alkaline hydrolysis is given in Table 15.

Oxidative degradation: The drugs were found to be slightly labile to oxidative degradation. After applying 3% hydrogen peroxide (H_2O_2) at room temperature (25 ± 2^0C) for one hour, about 16.17% degradation was observed. Additional two degradation peaks are observed at R_f 0.21 and 0.29 (Figure 10). Data of oxidative degradation is given in Table 16.

Photo degradation: When drug solution was exposed to direct sunlight for 3 hr, 35.63 % degradation was observed. Three additional peaks were observed at R_f 0.23, 0.39 and 0.54 (Figure 11). Data of photo degradation is given in Table 17 and 18.

Conclusion

Based on the results, obtained from the analysis using described method, it can be concluded that the method has linear response in the range of 200-700 ng/spot for FA and 40000 – 140000 for MCZ.

The result of the analysis of pharmaceutical formulation by the proposed method is highly reproducible and reliable and is in good agreement with label claim of the drugs. The additive usually present in the pharmaceutical formulations of the assayed samples did not interfere with MCZ and FA. The method can be used for the routine analysis of MCZ and FA in combined dosage form.

The study involved observation on degradation products formed under different stress condition. The developed method successfully separated drug substances from degradation products formed under various stress conditions. MCZ and FA are prone to alkaline hydrolysis and photolysis.

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