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Forced degradation studies and validated stability – indicating HPTLC method for determination of miconazole nitrate in soft lozenges

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ABSTRACT

Recently, Polyethylene glycol (PEG) has potentially been utilized as a matrix component prepared with fusion and mold method as an inert carrier to improve the solubility of poorly water-soluble drugs. [1, 2, 3] Miconazole Nitrate Soft Lozenges were prepared by Melting and Mold method and investigated for various analytical parameters. A New, Simple, Precise, Rapid and Selective Stability – Indicating High - Performance Thin - Layer Chromatographic (HPTLC) method for the quantification of Miconazole nitrate (MCN) in the prepared Soft Lozenges has been developed. It was performed on silica gel 60 GF_{254} Thin Layer Chromatographic plates using mobile phase comprising of Toluene: Chloroform: Acetonitrile: Ammonia (4.5:4:2.5:0.2 v/v/v/v) and the detection was carried out at 226 nm using densitometer. The Retention Factor (R_f) was found to be 0.45 for MCN. The linear regression analysis data for the calibration plots showed good linear relationship with r^2 = 0.9982 in the concentration range 4 - 60 µg /spot with respect to peak area. According to the ICH guidelines the method was validated for Linearity, Specificity, Recovery, Precision, Robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ). Forced Degradation Studies were carried out on Miconazole Nitrate as per ICH Guidelines. [4] Statistical analysis of the data showed that the method is reproducible and selective for the estimation of Miconazole Nitrate.

Keywords: HPTLC, Miconazole Nitrate, Soft Lozenges, Method validation

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INTRODUCTION

Miconazole Nitrate (MCN), or 1-[2, 4-dichloro-β-[(2, 4-dichlorobenzyl) oxy] phenethyl] imidazole, is an antifungal of the class of Imidazole. Miconazole Nitrate (Figure 1) is indicated in the treatment for various fungal infections such as infection of skin and nails, vulvo-vaginal candidosis, oral and esophageal candidosis and fungal colonization of GIT. [5, 6]

Various methods are available for the quantitative determination of Miconazole Nitrate such as HPLC, Gas chromatography, spectrophotometric methods such as UV spectrophotometry, Colorimetry and other methods such as Voltammetry. [7, 8] These reported methods have been used for the determination of Miconazole Nitrate individually or in combination with other drugs in different dosage forms such as tablets, creams, gels etc. However, there is no HPTLC method available for the estimation of Miconazole Nitrate in Soft Lozenges. HPTLC is a method that meets all quality requirements of today's analytical labs even in a fully regulated environment. Sophisticated instruments, controlled by an integrated software platform ensure to the highest possible degree the usefulness, reliability, and reproducibility of the data generated by High Performance Thin-Layer Chromatography (HPTLC).

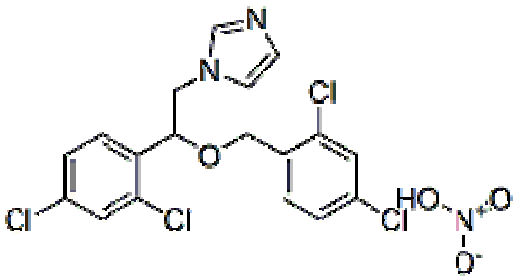

Figure 1: Molecular Structure of Miconazole Nitrate

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. Suceptibility to oxidation is one of the required tests. Also, hydrolytic and photolytic stability are required. An ideal stability-indicating method is one that quantitates the drug, per se, and also resolves its degradation products.

The aim of the present work is to develop an Accurate, Specific, Repeatable, and Stability - Indicating HPTLC method for the determination of Miconazole Nitrate in the presence of its degradation products for the assessment of purity of bulk drug and stability of the developed Soft Lozenges, using Toluene: Chloroform: Acetonitrile: Ammonia (4.5:4:2.5:0.2 v/v/v/v) as mobile phase on silica gel 60 GF_{254} TLC plates (Merck). Quantitative estimation was accomplished by densitometric scanning with UV detector at 226 nm wavelength. The method was successfully applied to the dosage form developed.

MATERIALS AND METHODS

Material and Chemicals

Pharmaceutical grade of Miconazole Nitrate was kindly supplied as gift sample by Cipla Ltd., Mumbai. All the chemical and reagents were of analytical grade and were supplied by S. D. Fine Chemicals Ltd., Mumbai, India. Soft Lozenges of Miconazole Nitrate developed in the Research Laboratory of Bharati Vidyapeeth's College of Pharmacy, C.B.D., Belapur were used for determination and quantification of drug.

Instrumentation

The HPTLC system consisted of a CAMAG Linomat 5 automatic spotting device, densitometric analysis was carried out utilizing CAMAG TLC scanner - 3, and these are supported with CAMAG WinCATS software. CAMAG twin-trough chamber (10 cm × 10 cm) was used. A 100 µl HPTLC syringe was used for application of samples. Sample application was done on precoated silica gel 60 F_{254}. (Merck, Germany).

Method

Preparation of Miconazole Nitrate Standard Solution

The standard solution was prepared by dissolving 40 mg of Miconazole Nitrate in sufficient amount of methanol and making up the volume up to 10 ml with it in a 10 ml volumetric flask. This stock solution had a concentration of 4000 µg/ml. This solution was used as working standard for analysis of all the samples.

Preparation of Sample Solution

Three Soft Lozenges (Figure 2) containing known amounts of Miconazole Nitrate (50 mg each) were weighed individually, and crushed and triturated uniformly in a mortar. Powder equivalent to 40 mg of Miconazole Nitrate was weighed and dissolved in sufficient amount of methanol in a 10 ml volumetric flask. The volume was made up to 10 ml with methanol and the flask was sonicated for 20 mins to extract the drug. The solution was then filtered, and the filtrate was used as the sample or test solution.

Chromatographic Conditions

Analysis was performed on 10 cm × 10 cm HPTLC silica gel 60 F_{254} plates. Sample and standard zones were applied to the layer as bands by means of a CAMAG. Linomat 5 automated spray - on applicator equipped with a 100 µl syringe and operated with the settings as band length 6 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 10 mm. The chromatographic development was performed using a mixture of

Toluene: Chloroform: Acetonitrile : Ammonia (4.5:4:2.5:0.2 v/v/v/v) as mobile phase for development under the following conditions, chamber saturation time, 10 min; temperature, 25 ± 2°C; migration distance, 80 mm. After development, the TLC plates were dried. Quantification of Miconazole Nitrate was achieved by scanning with CAMAG TLC scanner 3 (slit dimension, 5 mm × 0.45 mm; wavelength of determination, 226 nm at absorbance/reflectance mode using D_2 lamp, scanning speed, 10 mm/s). The chromatogram was plotted as absorbance against R_f values.

Calibration Plots

A series of standard curves were prepared over a concentration range of 4 - 60 µg. The data of area under the peak versus drug concentration was treated by linear least square regression analysis.

Method Validation [9, 10, 11]

The developed method was validated as per ICH guidelines. The developed method was validated in terms of Linearity, Specificity, Recovery, Precision, Robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ).

Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for Miconazole Nitrate in sample was confirmed by comparing the retention factor (R_f) and spectra of the spot with that of standard.

Linearity

Linearity of method was carried out by using standard solution of Miconazole Nitrate in concentration range of 4 - 60 µg/spot. The data of area under the peak versus drug concentration was treated by linear least square regression analysis.

Precision

Interday precision

Interday variations for determination of Miconazole Nitrate were carried out at three different concentration levels in triplicate - 10, 30, 50 µg/spot at three different days.

Intraday precision

Intraday precision for determination of Miconazole Nitrate were carried out at three different concentration levels in triplicate - 10, 30, 50 µg/spot on same day. Repeatability is also termed intra-assay or intraday precision. The reproducibility of sample application by measurement of concentration with respect area for MCN was expressed in terms of SD and % RSD for intraday and interday precision.

Recovery

Recovery studies were carried out by applying the method to a placebo sample (blank lozenge) to which the known amount of miconazole nitrate had been spiked. Recovery study was performed by spiking 80%, 100% and 120% amount of standard drug externally to the placebo samples. The experiment was conducted in triplicate. This was conducted to check the recovery of drug at different levels. Two bands of standard and three bands of each sample solutions (placebo samples spiked with standard MCN - 5 µl each) i.e. 20 µg/spot were applied on TLC plate and the plates were developed and scanned under the optimized chromatographic conditions.

Robustness

By introducing small changes in the mobile phase composition and saturation time the effects on the results were examined. The robustness of the method was performed by allowing variation in chamber saturation time by 8, 10, 12 min. and variation in mobile phase proportion - (4.3:4:2.7:0.2), (4.5:4:2.5:0.2), (4.7:4:2.3:0.2)

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were experimentally verified by diluting known concentrations of Miconazole Nitrate until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

Analysis of the Soft Lozenge Formulation
Two bands of the standard solution and three of sample solution 5 µl each were applied on TLC plates to give concentration of about 20 µg/spot for MCN. The plate was developed and scanned under the previously mentioned optimized chromatographic conditions. The amount of MCN present in the applied volume of sample was displayed by the instrument by comparing peak area of the sample with that of standard.

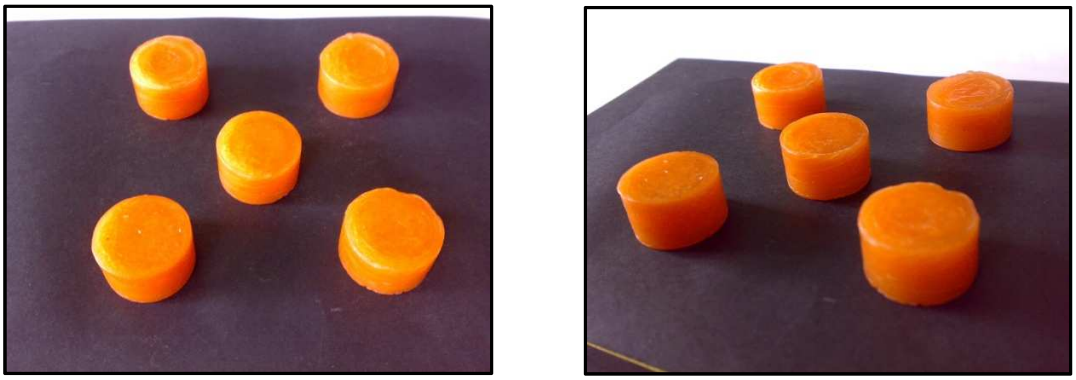

Figure 2: Soft Lozenges of Miconazole Nitrate

Forced Degradation of Miconazole Nitrate
A stock solution containing 40 mg Miconazole Nitrate in 10 ml methanol was prepared in a 10 ml volumetric flask. This solution was used for forced degradation.

Preparation of Acid and Base Induced Degradation Product
For acid and alkaline degradation studies, to 10 ml of methanolic stock solution, 10 ml each of 1N HCL and 1N NaOH were added separately. These mixtures were refluxed for 2.0 h at 80°C. 5 µl of the resultant solutions (20 µg/spot) were applied on the TLC plate, and the chromatograms were run as described previously.

Preparation of Hydrogen Peroxide Induced Degradation Product
To 10 ml of methanolic stock solution, 10 ml of Hydrogen Peroxide 6.0% w/v was added and then refluxed for 2.0 h at 80°C. 5 µl of the resultant solutions (20 µg/spot) were applied on the TLC plate, and the chromatograms were run as described previously.

Dry and Wet Heat Degradation Product
The powdered drug was stored in an oven at 80°C for 6.0 h to study Dry heat degradation, and the stock solution was refluxed at 80°C for 2.0 h in a water bath for Wet heat degradation. 5 µl of the resultant solutions (20 µg/spot) were applied on the TLC plate, and the chromatograms were run as described previously.

Photochemical Degradation Product
The photochemical stability of the drug was also studied by exposing the stock solution to direct sunlight for 48 h. 5 µl of the resultant solutions (20 µg/spot) were applied on the TLC plate, and the chromatograms were run as described previously. In all degradation studies, the average peak area of Miconazole after application of three replicates was obtained.

RESULTS AND DISCUSSION

Chromatography
In this study the quantitative HPTLC method was developed for the estimation of Miconazole Nitrate in the developed Soft lozenges. Various blends of solvent systems in varying proportions were tried as mobile phase. However, the solvent system comprising of Toluene: Chloroform: Acetonitrile: Ammonia (4.5:4:2.5:0.2 v/v/v/v) was found to give a good separation and resolution of Miconazole Nitrate without interference from the other materials. The peak area on the chromatogram was used for quantitative determination. During the development of the HPTLC method it was observed that a pre-saturation of the TLC chamber with mobile phase for at least 10 min

was required to obtain a good separation with reproducible R_f values. The R_f value of Miconazole Nitrate was found to be 0.45 ± 0.05.

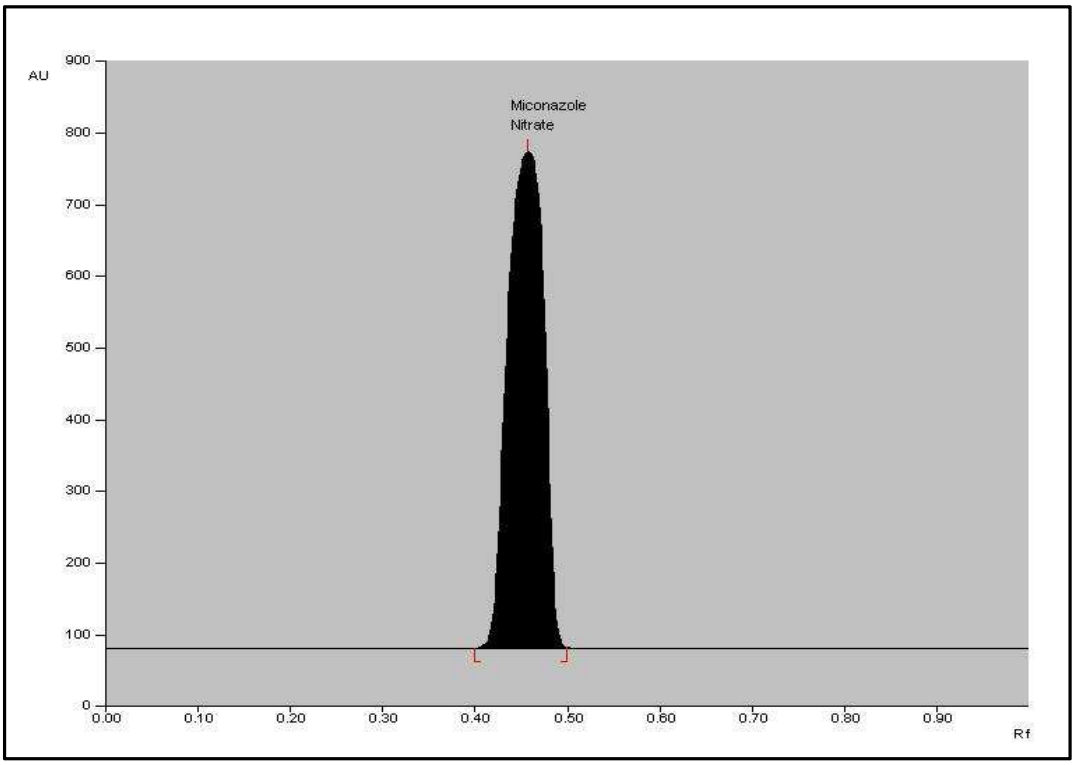

Figure 3: Densitogram of Standard Miconazole Nitrate (20 µg/spot), R_f = 0.45 ± 0.05, Mobile Phase - Toluene: Chloroform: Acetonitrile: Ammonia (4.5:4:2.5:0.2 v/v/v/v)

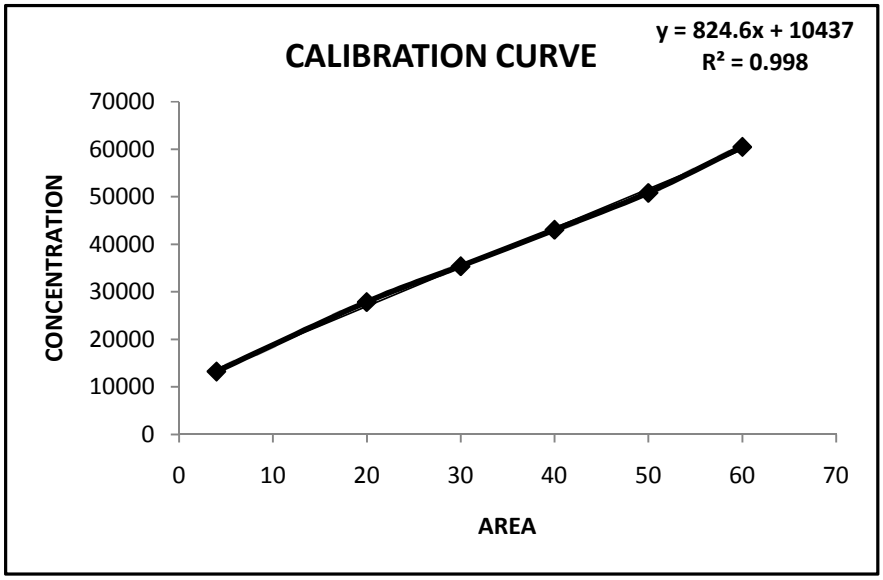

Figure 4: Calibration Curve of Miconazole Nitrate

Calibration curve

The polynomial regression data for the calibration plots showed a good linear relationship over concentrations range of 4 – 60 µg. No significant difference was observed in the slopes of standard curves (Table 1, Figure 3 and Figure 4).

The correlation coefficient r^2 was found to be;

$y = 824.6 x + 10437$

$r^2 = 0.998$

Table 1: Linear Regression Data for the Calibration Curves

Linearity range (µg per spot)	r^2 ± S.D.	Slope ± S.D.	Intercept ± S.D.
4 - 60	0.998 ± 0.0080	824.6 ± 88.12	10437 ± 54.94

Specificity

Good correlation was also obtained between Standard and Sample spectra and R_f of Miconazole Nitrate. The results are depicted in Figures 3 and 6 and, Figure 5 for comparison of Densitograms and Spectras of Standard and Sample respectively.

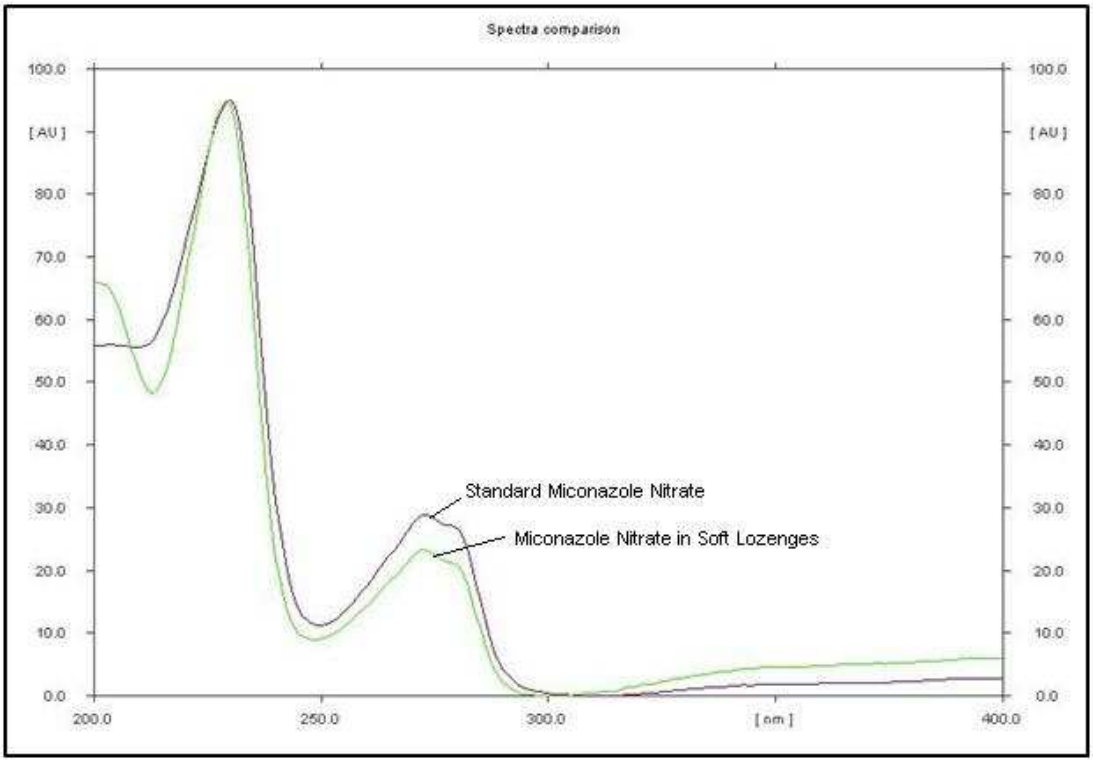

Figure 5: Ultraviolet Spectrum of Standard and Sample (Soft Lozenge) Miconazole Nitrate

Table 2: Recovery Studies on Miconazole Nitrate

Amount spotted (µg)	Peak area ± SD (AU)	Amount Recovered ± SD (µg)	Accuracy ± SD (%)	Mean Accuracy (%)	% R.S.D	Mean % RSD
4	13650.87 ± 50.32	4.02 ± 0.06	100.42 ± 1.52		1.51	
5	14483.33 ± 39.20	5.02 ± 0.05	100.46 ± 0.95	100.11	0.94	0.90
6	15265.77 ± 61.55	5.97 ± 0.07	99.47 ± 1.24		1.25	

Recovery Studies

The proposed method was used for extraction and subsequent estimation of Miconazole Nitrate from the developed Soft Lozenges. For the purpose of recovery studies, a blank Soft Lozenge (without MCN) was extracted in the same manner mentioned previously for the lozenge containing MCN. This sample solution of blank lozenge was spiked

with known amounts of MCN. After spiking with the drug (80%, 100% and 120%), the proposed method afforded recovery in the range of 98.59 – 101.91% for MCN from the Soft Lozenges (Table 2).

Precision

The reproducibility of sample application by measurement of concentration with respect to area for MCN was expressed in the terms of SD and %RSD and the results are shown in Table 3. It was observed that, the percentage coefficient of variation of intraday precision and interday precision was comparable and within the limits.

Table 3: Precision Studies on Miconazole Nitrate

	Amount spotted (µg)	Mean Peak area ± SD (AU)	Mean Amount Recovered ± SD (µg)	% R.S.D.	Mean % RSD
Interday	10	18620.07 ± 253.30	10.02 ± 0.39	1.58	
	30	35576.03 ± 375.29	30.50 ± 0.45	1.31	1.22
	50	51656.97 ± 43.76	49.93 ± 0.05	0.12	
Intraday	10	18815.73 ± 126.24	10.25 ± 0.15	0.91	
	30	35806.03 ± 504.15	30.38 ± 0.34	1.81	1.00
	50	51369.40 ± 340.58	49.99 ± 0.33	0.96	

Robustness

The SD of peak areas was calculated for each parameter (mobile phase composition and saturation time) and %RSD was found to be less than 2%. The developed method was found to be robust as the changes in peak areas and R_f values were within the limits after varying saturation time and mobile phase proportion (Table 4 and Table 5).

Table 4: Variation in R_f And Peak Area with Change in Composition of Mobile Phase

Sr. no.	Saturation time in min	R_f ± SD	Mean Peak Area ± SD (AU)	% RSD	Mean % RSD
1	8	0.45 ± 0.02	25996.18 ± 448.67	1.72	
2	10	0.45 ± 0.02	26036.25 ± 420.56	1.61	1.60
3	12	0.45 ± 0.02	25934.61 ± 382.52	1.47	

Table 5: Variation in R_f and Peak Area with Change in Saturation Time

Sr. no.	Mobile phase proportion	R_f ± SD	Mean Peak Area ± SD (AU)	% RSD	Mean % RSD
1	4.7:4:2.3:0.2	0.45 ± 0.05	25154.50 ± 407.15	1.61	
2	4.5:4:2.5:0.2	0.45 ± 0.05	26036.25 ± 420.56	1.61	1.70
3	4.3:4:2.7:0.2	0.45 ± 0.05	25121.95 ± 382.52	1.80	

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The s/n of 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 219 ng and 666 ng/spot, respectively (Figure 7 and Figure 8).

Analysis of the Developed Soft Lozenge Formulation

A single spot at R_f of 0.45 (Figure 6) was observed in the chromatogram of the drug samples extracted from the Soft Lozenges. There was no interference from the excipients present in the Soft Lozenges. The drug content was found to be 101.06 ± 2.5 with a %RSD of 1.38. It may therefore be concluded that degradation of Miconazole Nitrate had not occurred in the lozenge formulation that were analyzed by this method. The low %RSD value indicated the suitability of this method for routine analysis of Miconazole Nitrate in pharmaceutical dosage form (Table 6).

Table 6: Results of Analysis of Soft Lozenges of Miconazole Nitrate

Formulation	Drug	Label Claim	Drug Content (%)	% RSD
Soft Lozenges	Miconazole Nitrate	50 mg	101.06 ± 2.5	1.38

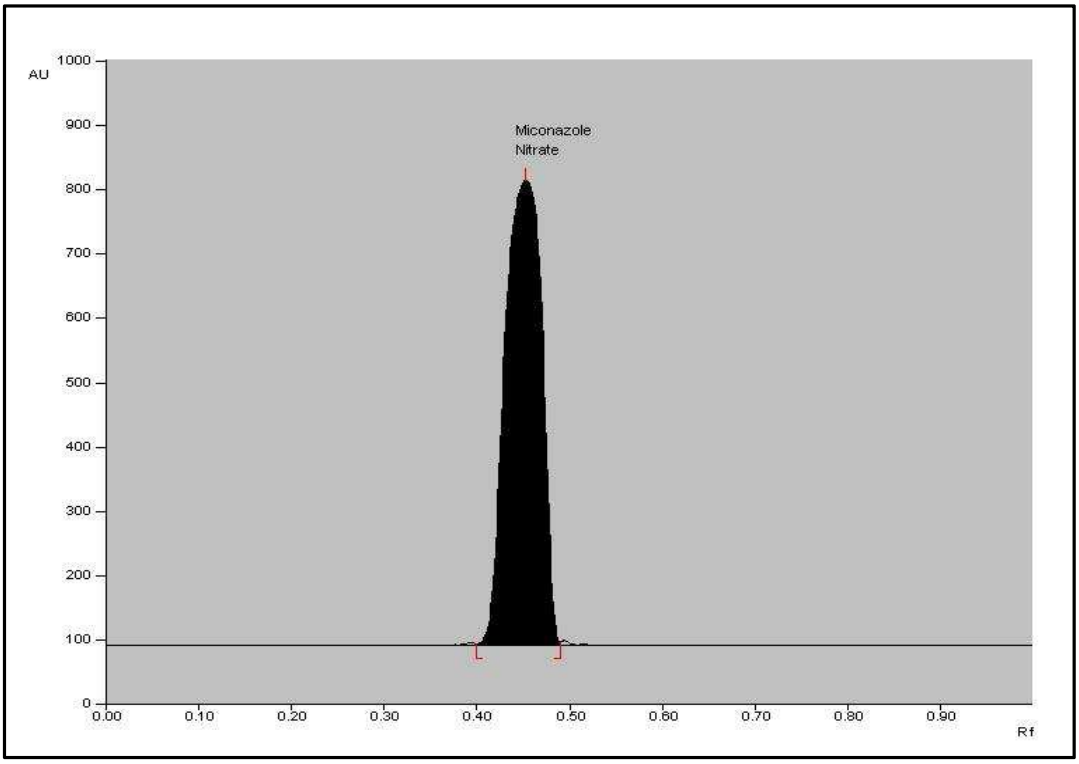

Figure 6: Densitogram of Sample (Soft Lozenge) Miconazole Nitrate (20 µg/spot), R_f = 0.45 ± 0.05, Mobile Phase - Toluene: Chloroform: Acetonitrile: Ammonia (4.5:4:2.5:0.2 v/v/v/v)

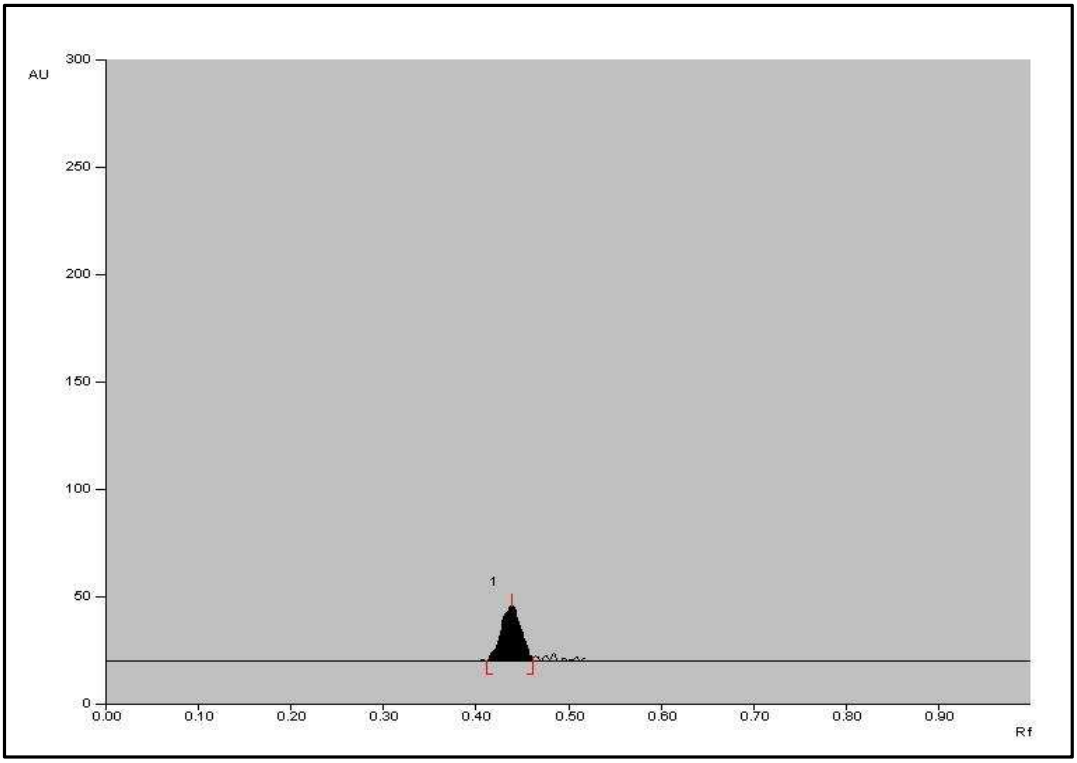

Figure 7: Densitogram of Miconazole Nitrate (Limit of Detection: 210 ng/Spot): Peak 1, Miconazole Nitrate

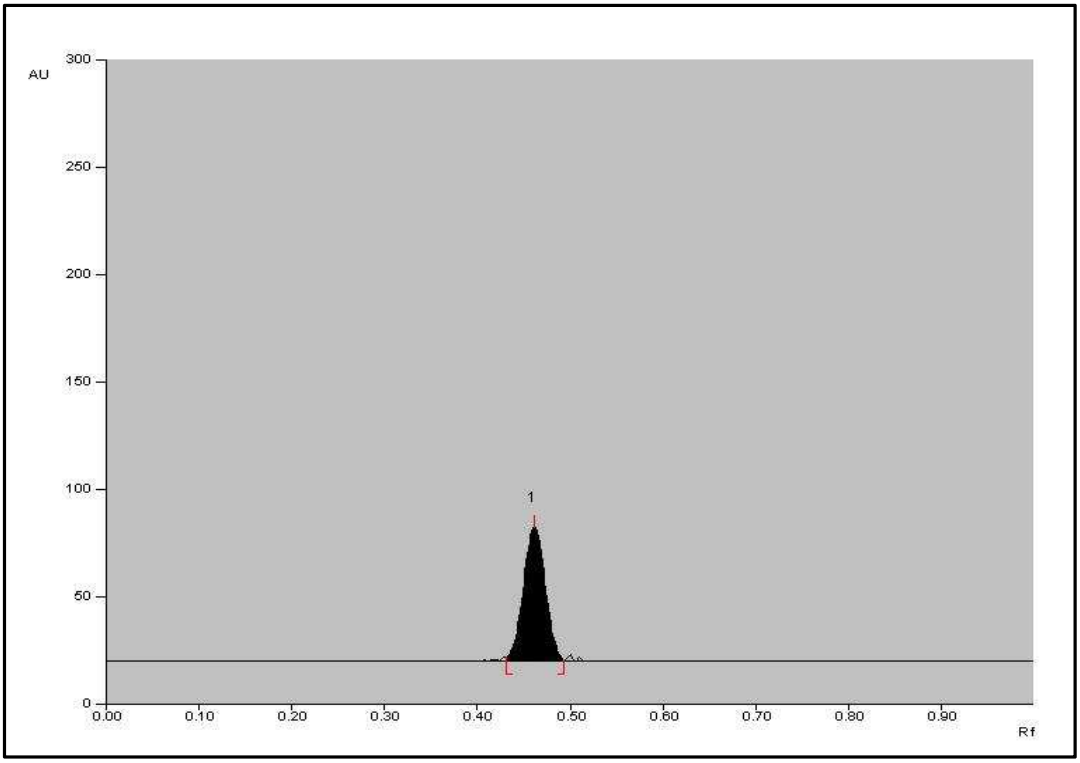

Figure 8: Densitogram of Miconazole Nitrate (Limit of Quantification: 660 ng/Spot): Peak 1, Miconazole Nitrate

Forced Degradation Studies

Acid and Base Induced Degradation Product

The chromatogram of the Acid degraded sample for Miconazole Nitrate showed three peaks at R_f value of 0.13, 0.2 and 0.23 respectively (Figure 9). The areas of the degraded peaks were found to be less than the area of the standard drug concentration (20 µg/spot), indicating that MCN undergoes degradation under acidic condition. The chromatogram of the Base degraded sample (Figure 10) showed degraded peaks at more or less same R_f values at that seen in acid degradation and additional small peaks at R_f 0.1 and 0.35. In both the cases the typical drug peak nature was missing (reduction in height as well as in area and broader peak base) without a corresponding rise in a new peak. This concluded that the drug was hydrolyzed under acidic and basic conditions to degraded chromophoric products.

Hydrogen Peroxide Induced Degradation Product

Degradation of Miconazole Nitrate occurred in hydrogen peroxide, showing various small degradation peaks in the R_f range of 0.2 – 0.7. A shift in the R_f was also observed after degradation from 0.45 to 0.41. The spots of degraded product were well differentiated from the drug spot (Figure 11).

Dry and Wet Heat Degradation Product

The samples subjected under Dry and Wet heat conditions did not show an additional peak. This indicated that MCN did not degrade in dry and wet heat conditions.

Photochemical Degradation Product

The Photo degraded sample showed no additional peak when drug solution was left in daylight for 48 h. This indicates that the drug is susceptible to Acid - Base hydrolysis and Oxidation.

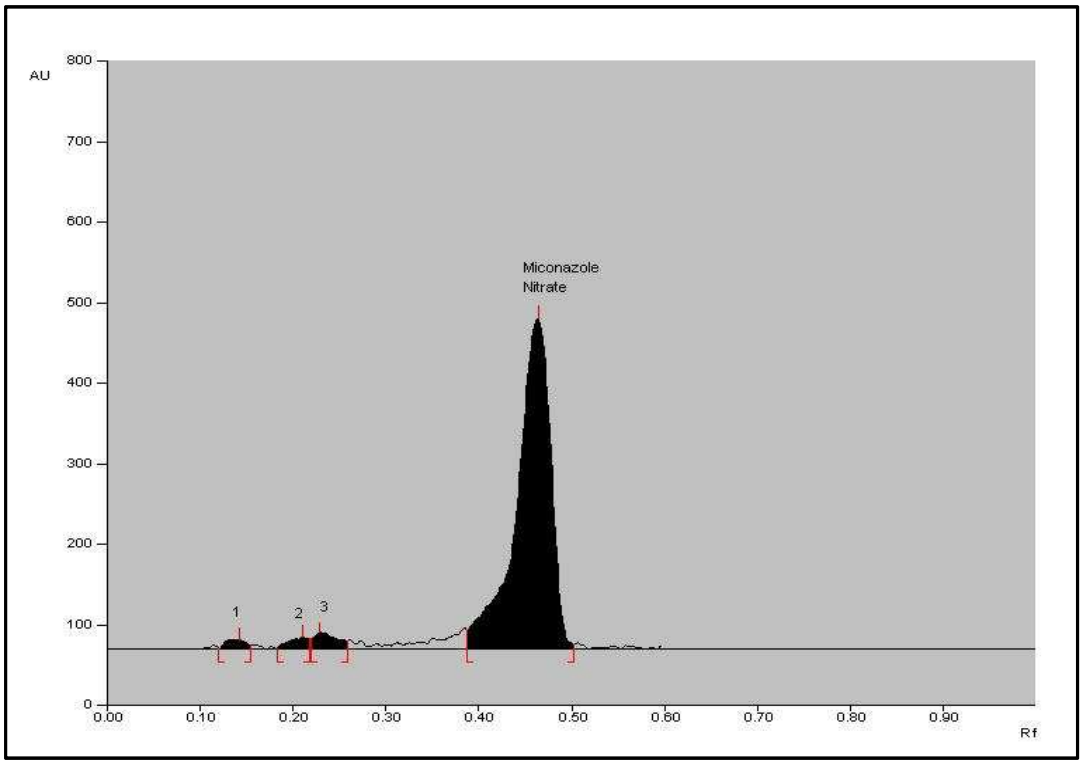

Figure 9: Densitogram Of Acid Treated Miconazole Nitrate (20 µg/spot): Peak 1, Degradant (R_f = 0.13); Peak 2, Degradant (R_f = 0.2); Peak 3, Degradant (R_f = 0.23); Peak 4, Miconazole Nitrate (R_f = 0.45)

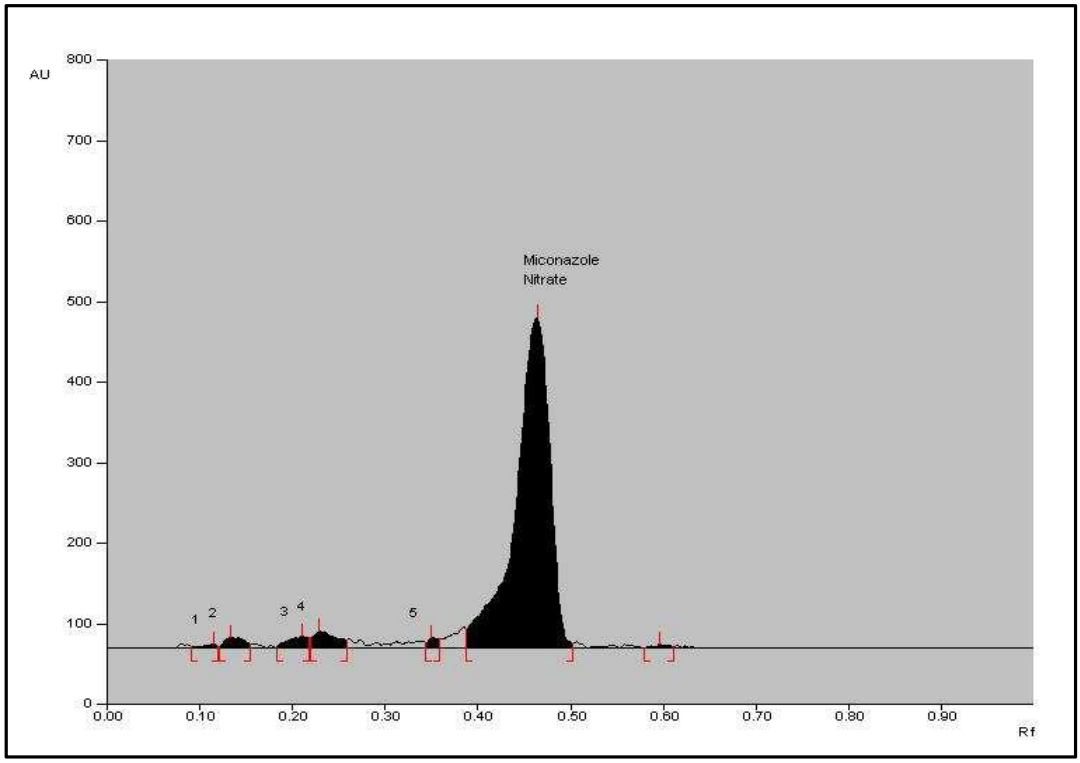

Figure 10: Densitogram of Base treated Miconazole Nitrate (20 µg/spot): Peak 1, Degradant (R_f = 0.11); Peak 5, Degradant (R_f = 0.36); Other Degradation Peaks similar to Acid Degradation Peaks; Peak 6, Miconazole Nitrate (R_f = 0.45)

Table 7: Summary of Validation Parameters

Parameter	Data
Linearity Range	4 – 60 µg/spot
Correlation Coefficient	0.998 ± 0.008
Limit of Detection (n = 6)	219 ng/spot
Limit of Quantification (n = 6)	666 ng/spot
Recovery (n = 9)	100.11 ± 0.90
Precision (% RSD) Interday (n = 9) Intraday (n = 9)	 1.22 1.00
Robustness	Robust
Specificity	Specific

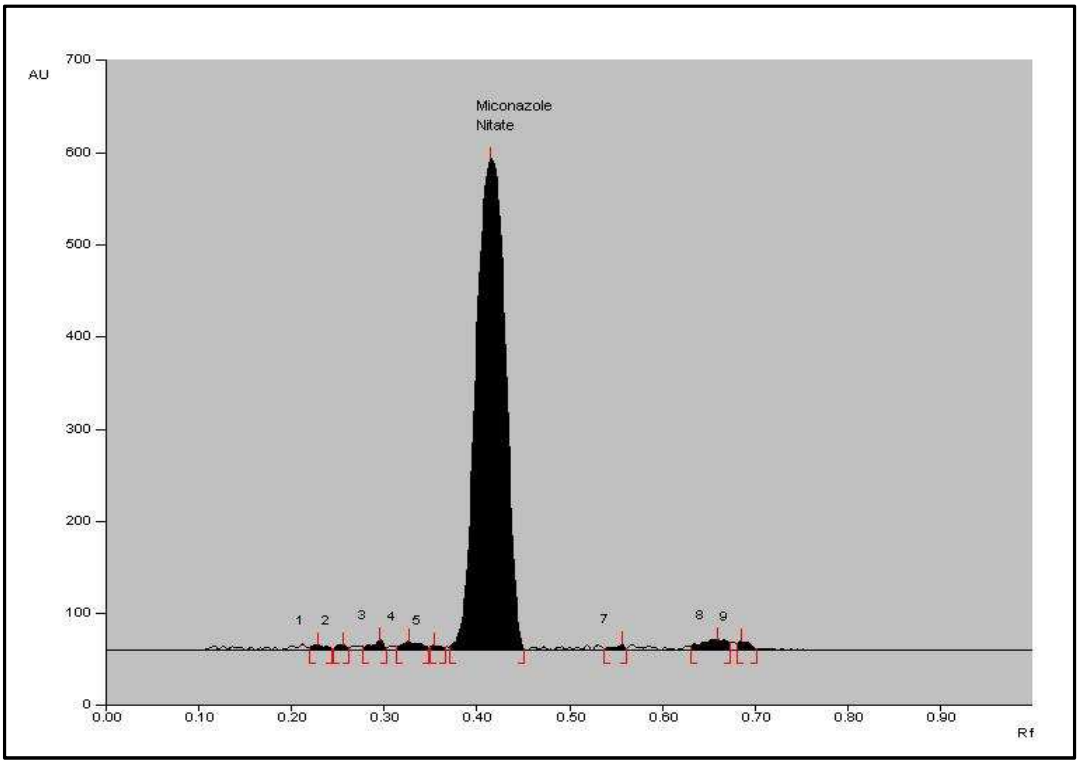

Figure 11: Densitogram of Hydrogen Peroxide treated Miconazole Nitrate (20 µg/Spot): Degradation Peaks in R_f Range 0.2 – 0.7; Peak 6, Miconazole Nitrate (R_f = 0.41)

CONCLUSION

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance. This powerful and adaptable technology now occupies a pivotal position ensuring the identity, purity, concentration conformity, and physicochemical stability studies of various pharmaceutical dosage forms. The HPTLC CAMAG device is now one of the cornerstones of our quality assurance system. This analytical tool represents an undeniable contribution to accreditation and certification procedures to which quality control organizations are now committed in order to improve the quality of analytical method development. [12, 13]

The Stability – Indicating method was developed on HPTLC for Miconazole Nitrate in Soft Lozenge formulation, for the first time in order to analyze more samples in less time. The proposed method is easy to perform, precise, accurate, rapid and reasonably specific and rugged. It is one of the rare studies in which forced degradation was carried out under all different suggested conditions. The whole procedure may be extended to pharmaceutical preparations and other applications on the same drug for routine screening without any interference from the excipients.

The presented method is an affirmation of both the effectiveness and ecological quality of modern instrumental TLC. As a result of the timely combination of a traditional method and spectrometry, computer-aided technologies, and qualitative as well as quantitative modern planar chromatography are rapidly gaining acceptance throughout the laboratories.

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