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RESEARCH ARTICLE

SIMULTANEOUS DETERMINATION OF CLOTRIMAZOLE AND TINIDAZOLE IN TABLET AND CREAM BY HPTLC

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Abstract

The present work describes a normal phase HPTLC method for simultaneous determination of clotrimazole and tinidazole in tablet and cream. The developed method is more sensitive than the reported method. Chromatographic separation was carried out on aluminum-backed silica gel 60 GF_{254} TLC plates with mobile phase comprising of toluene: ethyl acetate: methanol: triethyl amine (**5.5:1.0:1.0:0.1, v/v**). The validated calibration ranges were 200-700 ng/spot (r=0.9960 and 0.9960 by height and area respectively) and 500-1750 ng/spot (r=0.9990 and 0.9975 by height and area respectively) for clotrimazole and tinidazole respectively. Quantitation was achieved with UV detection at λ=220 nm. The method was validated in terms of accuracy, precision and specificity. The method has been successfully applied to pharmaceutical formulations. Interference was not observed from the tablet and cream formulations excipients.

Introduction

Clotrimizole (CTZ) is an antifungal agent. Survey of literature revealed various methods like supercritical fluid extraction and second order derivative spectroscopic (Bonazzi et al., 1998), indirect titrimetric and extractive-spectrophotometric (Farhadi and Maleki, 2002), colorimetric (El-Shabouri et al., 1998; Kelani et al., 1997), HPLC (Gagliardi et al., 2003; Abdel Moety et al., 2002), spectrophotometric (Khashaba et al., 2000), TLC (Cakar et al., 2005; Roychowdhury and Das, 1996) and stripping voltametric (Pereira et al., 2001) for the determination of clotrimazole in formulations. Similarly colorimetric (Sinngbal and Kuchadkar, (1988), spectrophtometric (Prasad et al., 1999; Trivedi and Sachan 1999; More et al., 1994; Shrinivas et al., 1999; Gupta et al., 2000; Gandhimati, 2000; Mahadik and Panzade, 2001; Jadhav and Arbad, 2004; Tipre and Kasture 2000) and HPLC (Halkar and Ankalkope 2000; Bakshi and Singh, 2004), methods have been reported for the determination of tinidazole (TNZ) alone or in combination with other drugs.

Derivative spectrophotometric (Prasad et al., 1997) and RP-HPLC (Tendulkar et al., 1994) methods are reported for simultaneous determination of clotrimazole and tinidazole in a combined dosage form. Both the methods do not report validation of the method.

Survey of literature also revealed a HPTLC (Vaidya et al., 2007) method for simultaneous determination of clotrimazole and tinidazole from tablet. In the reported method quantification was carried out at 254 nm, the wavelength at which sensitivity for clotrimazole is decreased. Further in this reported paper, fig. 2 depicting chromatograms of clotrimazole and tinidazole is contradictory, as at 254 nm, tinidazole has much more UV-absorption as compared to clotrimazole and further the concentration of tinidazole in the formulation is five times that of clotrimazole.

The present work presents a better method for simultaneous determination of clotrimazole and tinidazole in tablet and cream using HPTLC-densitometry. The method does not require any prior extraction of the two drugs for determination from cream. The method is simple and reduces the duration of analysis and suitable for routine determination of the two drugs.

2. Experimental

2.1 Materials and Chemicals

Pharmaceutical grade of clotrimazole (CTZ) and tinidazole (TNZ) were kindly supplied by Nestor Pharmaceuticals Ltd., Faridabad, India as gift samples. All chemicals and reagents were of HPLC grade and were purchased from Merck Chemicals, India. The two formulations Ginal-V tablet (TNZ-500 mg and CTZ-200 mg) and Ginal-V cream (TNZ-5%w/w and CTZ-2% w/w) were purchased from a local pharmacy.

Normal phase aluminum backed silica gel 60 F_{254} TLC plates (Merck) were purchased locally.

2.2 Instrumentation and chromatographic conditions

The samples were spotted in the form of bands 4 mm wide with Camag 100µl sample syringe on aluminum-backed silica gel 60 GF_{254} TLC plates. The plates were prewashed with methanol and activated at 110° for 10 min prior to chromatography. A constant application rate, 5 s/µl was employed and space between two bands was 4 mm. The sample was applied with the help of Camag Linomat IV automatic sample applicator. The mobile phase comprised of toluene: ethyl acetate: methanol: triethyl amine (5.5:1:1:0.1, v/v/v/v). Linear ascending development was carried out in twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was 10 min at room temperature (25°C±2) at relative humidity of 60%±5. For saturation, filter papers previously soaked in mobile phase were used on the two large sides of the chamber. The length of chromatogram run was 7 cm. Subsequent to the development; TLC plates were dried in a current of dry air with the help of an air dryer.
Densitometric scanning was performed on Camag TLC scanner III in reflectance-absorbance mode at λ= 220 nm for all measurements and operated by CATS 4.0 software. The source of radiation utilized was deuterium lamp. Evaluation was via peak height and peak area.

2.3 Standard solution

Stock standard solution was prepared by dissolving 10 mg of clotrimazole and 25 mg of tinidazole in 10.0 ml of methanol. The standard solution was prepared by dilution of the stock standard solution with methanol to get concentration of 100 and 250 µg/ml for clotrimazole and tinidazole respectively.

2.4 Sample preparation

Tablet

To determine the content of clotrimazole and tinidazole simultaneously in tablet, twenty tablets were accurately weighed and average weight was calculated. The tablets were then crushed to fine powder; an accurately weighed quantity of tablet powder equivalent to about 10 mg of clotrimazole and 25 mg of tinidazole was transferred to 10.0 ml volumetric flask and dissolved with shaking in methanol for 15 min. The volume was made up to the mark with methanol and the solution was mixed, filtered and further diluted to get final concentration of 100 µg/ml of clotrimazole and 250 µg/ml of tinidazole.

Cream

For determination of clotrimazole and tinidazole in cream, an accurately weighed quantity of cream equivalent to 6.25 mg of tinidazole was transferred to a 25.0 ml volumetric flask and shaken with 15 ml methanol for 25 min with mild warming. The volume was made up to the mark with methanol. The solutions were then centrifuged and the supernatant was used as sample solution containing 100 µg/ml of clotrimazole and 250 µg/ml of tinidazole.
The sample solution (4µl) each, were applied on TLC plate to give concentration 400 ng/spot for clotrimazole and 1000 ng/ spot for tinidazole respectively. The plate was developed and scanned under the optimized chromatographic conditions. The peak height and area of the spots were measured at λ=220 nm for clotrimazole and tinidazole, respectively, and their concentration in the samples was displayed by the instrument by comparing peak height and area of the samples with that of standard.

3. Results and Discussion

3.1 Optimization of Mobile Phase

Various blends of solvent systems in varying proportions were tried as mobile phase. However mobile phase consisting of toluene: methanol: ethyl acetate: triethylamine (**5.5:1.0:1.0:0.1, v/v**) gave good resolution and dense and compact spots. Use of triethylamine helped to reduce peak tailing of tinidazole which is basic in nature. The detection wavelength was selected from overlain in-situ UV-spectra of clotrimazole and tinidazole. The selection of wave length was based on UV absorbance by both the drugs with due consideration to their ratio in the formulations (**Fig. 1**). The optimized chamber

saturation time with the mobile phase was 10 min at room temperature (25°C±2) at relative humidity of 60%±5. The length of chromatogram run was 7 cm. Both peaks were symmetrical in nature and no tailing was observed when scanned at λ= 220 nm **(Fig. 2)**.

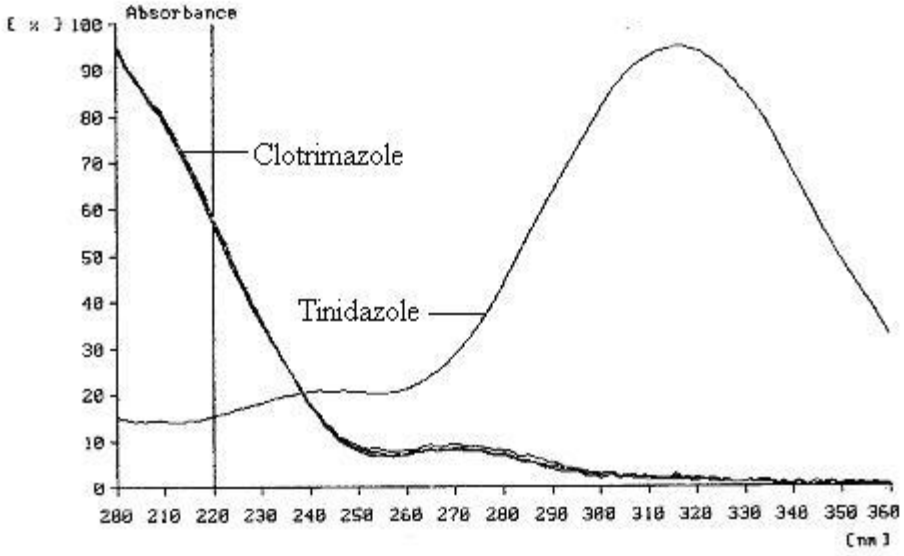


Fig.1. In-situ overlain spectrum of clotrimazole and tinidazole in absorbance and reflectance mode.

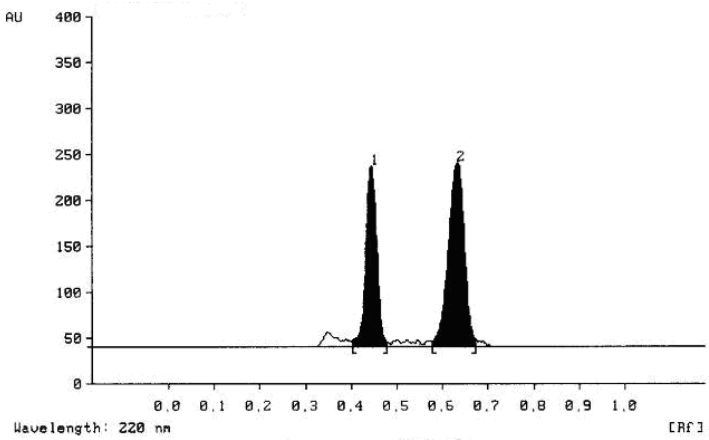


Fig.2. Densitograms of Tinidazole (1000 ng/spot); peak 1 (Rf: 0.45±0.02) and Clotrimazole (400ng/spot); peak 2 (Rf: 0.65±0.02) in standard solution.

3.2 Method Validation

The method was validated for linearity, precision, accuracy and specificity.

3.2.1 Linearity

Standard solutions were prepared by dilution of the stock standard solution with methanol to get concentration of 100 and 250 µg/ml for clotrimazole and tinidazole respectively. This standard solution was applied on the TLC plate in the range of 2-7 µl three times (500-1750 ng/spot for tinidazole and 200-700 ng/spot for clotrimazole). The plate was developed using previously described mobile phase and scanned under the optimized chromatographic conditions. Peak height and peak area were recorded for each drug concentration and the instrument displayed the linear regression curve for concentration vs. peak height/ area for both the drugs. A linear response was observed over the examined concentration range. Tinidazole showed good correlation coefficient in the concentration range of 500-1750 ng/spot (r = 0.9990 and 0.9975 by height and area respectively) and clotrimazole in the range of 200-700 ng/spot (r = 0.9960 and 0.9960 by height and area respectively). The linear regression data is given in **Table 1**. Apart from correlation coefficient value the intercept value was not more than 2% of the response observed at 100% concentration in case of peak height and peak area and hence, single point calibration was used.

Table 1: Linear regression data for calibration curves (n=3)

Parameter	Tinidazole		Clotrimazole	
	By Peak Height	By Peak Area	By Peak Height	By Peak Area
Linearity(ng/spot)	500-1750	500-1750	200-700	200-700
Correlation coefficient.	0.999	0.9975	0.9960	0.9960
Slope, ±S.D.	123.85, 1.1	1876.12, 9.34	331.53, 3.25	7984.14, 28.94
Y-Intercept, ±S.D.	67.63, 0.80	865.98, 6.11	48.99, 0.45	605.66, 4.52

3.2.2 Precision

The system repeatability (intra-day precision, %R.S.D.) was assessed from six determinations at 100% of the test concentration of sample solutions and standard solutions. The results are reported in **Table 2**. Intermediate precision is a measure of precision between repeatability and reproducibility. It is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory (ICH Harmonised Tripartite Guidelines, Q2B, 1997). Because these parameters influence the response together, it becomes necessary to study and observe such effects. In this case the factors considered were the analyst (analyst 1, analyst 2 and analyst 3) and the day (day 1, day 3 and day 5). Due to unavailability of another HPTLC unit, multiple instrument factors could not be studied. The results are reported in **Table 3.** The %R.S.D. values indicate that the proposed method provides acceptable inter-day and different analyst variation for determination of clotrimazole and tinidazole.

Table 2: Results of System repeatability

Drug	Concentration [ng/spot]	Intraday precision [%RSD, n=6] By Peak Height	By Peak Area
Clotrimazole	400	0.83	0.73
Tinidazole	1000	0.65	0.71

Table 3: Results from Study of Intermediate Precision

Parameter	Tinidazole		Clotrimazole	
	By Peak Height	By Peak Area	By Peak Height	By Peak Area
Analyst – 1 [% estimated]	98.08	101.60	100.26	98.77
Analyst – 2 [% estimated]	101.20	99.62	101.24	99.99
Analyst – 3 [% estimated]	100.48	99.80	101.29	99.97
Inter-day precision [%RSD, n=6]	0.88	0.82	0.79	0.71

3.2.3 Accuracy/Recovery studies

Recovery study was carried out by applying the method to drug sample to which the known amount of clotrimazole and tinidazole has been spiked (standard addition method). The final concentration of the mixture containing drug product and spiked drug was in the range of 80, 90, 100, 110 and 120% of the label claim.

The proposed method when used for extraction and subsequent estimation of clotrimazole and tinidazole from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98.77-100.49% and 98.50-101.01% for tinidazole and clotrimazole respectively and mean recovery for clotrimazole and tinidazole from marketed formulations are listed in **Table 4**.

Table 4: Result of Recovery Study (n=5)

	Tinidazole		Clotrimazole	
	By Peak Height	By Peak Area	By Peak Height	By Peak Area
Tablet				
% recovery ±S.D.	99.32±0.80	100.19±0.19	98.50±0.43	99.92±0.72
% R.S.D.	0.80	0.19	0.43	0.72
Cream				
% recovery ±S.D.	98.77±0.36	100.44±0.18	99.63±0.62	101.01±0.49
% R.S.D.	0.37	0.18	0.62	0.48

3.2.4 Specificity studies

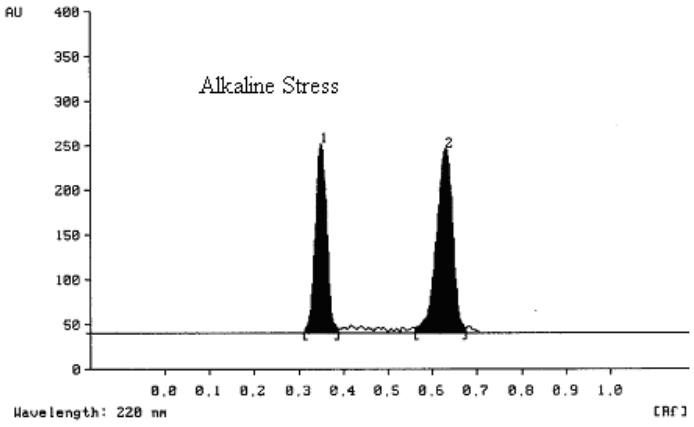
The specificity is the ability to access unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. Sample solution were prepared by exposing an accurately weighed quantity of tablet powder equivalent to 10 mg of clotrimazole and 25 mg of tinidazole to various stress conditions, like at 50° after addition of 1.0 ml of 0.1 N HCl (acid), at 50° after addition of 1.0 ml of 0.1 N NaOH (alkali), at 50° after addition of 1.0 ml of 3.0% H_2O_2 (oxide), at 60° and in UV-cabinet at 265 nm for 24 hr. After 24 h, the content in the flasks were shaken with 5-6 ml of methanol for 15 min. Rest of the procedure was same as described under sample preparation for tablet. The sample solutions were then analyzed by the proposed method.

The results of specificity studies indicate that tinidazole was completely degraded in basic condition. This was observed as a shifting of peak of tinidazole as degraded product in alkaline stressed sample **(Fig. 3A)** to a lower Rf value with respect to that of standard. Similarly in acidic stress condition both tinidazole and clotrimazole undergo degradation as observed by the decrease in peak height and peak area of both the drugs. An extra peak was observed at Rf value 0.35 which may be due to degradation of the two drugs **(Fig. 3B)**. Thus it can be inferred that, the mobile phase is able to separate degradation

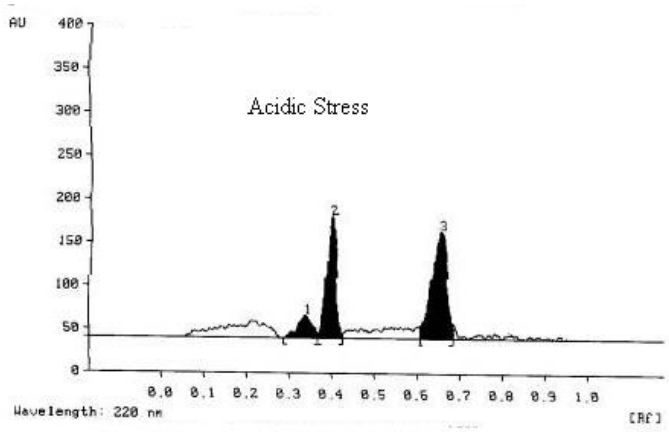
product from the target drugs and offer determination without interference of the degradation product. Both the drugs were found to be stable under oxide, heat and UV-exposure conditions as depicted by the assay values in **Table 5.** Any by-products of synthesis of CTZ and TNZ that may be present were not observed in the chromatograms studied. This indicated that any by-products of synthesis that may be present must be in negligible quantity,

Table 5: Results of Specificity Study-HPTLC

Stress Condition	Tinidazole (% estimated)		Clotrimazole (% estimated)	
	By Peak Height	By Peak Area	By Peak Height	By Peak Area
Acid	96.05	88.67	73.07	74.31
Alkali	----	----	98.55	100.96
Oxide	98.23	100.50	100.53	98.11
UV	100.61	98.78	98.93	99.05
Heat	100.72	100.22	98.07	99.54



a)



b)

Fig.3 Densitograms under Stressed Conditions

(a) Alkaline stress: peak 1 (Rf: 0.35) degradation product of tinidazole and peak 2 clotrimazole (Rf: 0.65),

(b) Acidic stress: peak 1 (Rf: 0.35) degradation product, peak 2 (Rf: 0.45) Tinidazole and peak 3 clotrimazole

(Rf: 0.65)

4. Analysis of Marketed Formulations

The spots at Rf 0.44 (TNZ) and 0.65 (CTZ) were observed in the densitograms of the drug samples extracted from tablet and cream. There was no interference from the common excipients present in tablet and cream. The assay values for both the drugs in formulations suggest that degradation of tinidazole and clotrimazole had not occurred in the marketed formulations that were analyzed by this method as shown in **Table 6**.

Table 6: Results of Estimation of Drugs in Marketed Formulations (n=5))

	Tinidazole		Clotrimazole	
	By Peak Height	**By Peak Area**	**By Peak Height**	**By Peak Area**
Tablet %estimated ±S.D.	100.05±0.77	99.62±0.56	100.23±0.85	99.46±0.81
Cream % estimated ±S.D.	100.34±0.99	100.18±0.81	100.60±0.74	100.01±0.71

5. Conclusion

The proposed HPTLC method provides good resolution of the target analytes with symmetrical chromatograms without tailing. The optimized mobile phase is also able to resolve degradation products from target analytes. Thus indicating the developed method is quite sensitive. The results of method validation depict the reliability of the method. The HPTLC method cited in reference no. 25, reports assay for tablet and not applied to cream and hence the claim in that paper is limited to tablet only. Further chromatograms reported in that method using the wavelength 254 nm are not justified looking at UV absorption of clotrimazole and tinidazole at that particular wavelength. Hence this is better method which can be applied for assay of tablet and cream.

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