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Chromatographic determination of clotrimazole, ketoconazole and fluconazole in pharmaceutical formulations

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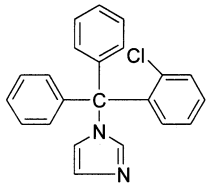
Abstract

High-performance liquid chromatographic technique has been developed for the determination or some azolcsantifungals namely, clotrimazole (CZ), ketoconazole (KZ) and fluconazole (FZ), in pure forms and in pharmaceutical formulations. The proposed HPLC-method can be successfully applied as a stability indicating method for the determination of CZ in presence of its acid degradation products; viz (2-chlorophenyl) -diphenyl methanol and imidazole. The analyzed drugs were separated on a reversed-phase column [Bondapak^{TM} C_{18} (10 μm, 25 cm × 4.6 mm, i.d.)] using a mobile phase containing acetonitrilc+25 mM trishydroxymeihyl aminomethane in phosphate butter (pH 7) = 55:45 (v/v), with UV-detection at 260 nm. The differences in the retention times (t_R) of the three azoles permit their use as internal standard for each other. In addition, a coupled TLC-densitometric method has been also applied as a stability indicating method to separate and quantify CZ alone or in presence of byproducts impurities and/or its acid degradation products. The TLC-fractionation was performed on a precoatcd silica gel F_{254} plates using a solvent system consisting of chloroform+acetone+ammonia (25%) (7:1:0.1, by volumes), CZ was well separated from its acid degradation products and quantified by densitometric scanning at 260 nm.

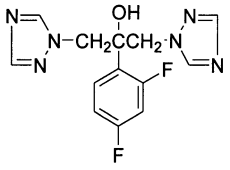
Keywords: Clotrimazole; Fluconazole; Imidazole; HPLC and TLC chromatography; Ketoconazole

1. Introduction

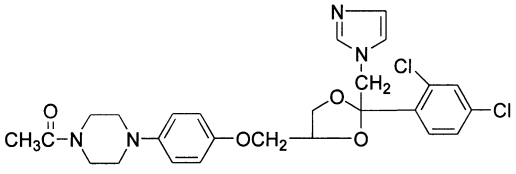
Clotrimazole, [1-[(2-chlorophenyl)diphenyl methyl]-1H-imidazole, is a topically used antifungal drug with broad spectrum antimicrobial activity. While ketoconazole; *cis*-l-acetyl-4[4-[2-(2,4-dichlorophenyl)-2-(111-imidazole-l-ylmethyl)-1,3-dioxolan-4-yl]methoxyphenyl] piperazine; and fluconazole; [α-(2-4-difluoro-phenyl]-α-[1H-1,2,4-triazole-1-ylmethyl-1H-l,2,4-triazole-1-ethanol]; are systemically used antifungal drugs in oral, esophagal and vaginal candidiasis [1,2].



Clotrimazole



Fluconazole



Ketoconazole

Clotrimazole, ketoconazole and fluconazole as antifungal drugs could be determined singles or in admixtures by several analytical techniques including; liquid

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chromatography [3–8], TLC–densitometry [9,10], gas–liquid chromatography (GLC), [11,12], spectrophotometry [13–16], electrochemically [17,18], pH indicator absorbance ratio method [19], titrimetry [20], and supercritical-fluid chromatography (SFC) with UV-detection [21].

The spectral data obtained by NMR, MS, IR, spectrometry could be used for characterization of fluconazole [22].

Clotrimazole is stable in alkaline medium but hydrolyzes in acid medium to (2-chlorophenyl)-diphenyl methanol and imidazole [23] (Scheme 1).

Stability of ketoconazole in ethanolic solutions was studied at room temperature or at 8 °C for 29 days [24].

Thermal stability of KZ film was confirmed by reversed phase HPLC, the calculated half-life and shelf-life were found to be 11.4 and 1.7 years, respectively [25]. Fluconazole may be also considered chemically stable over the studied storage conditions of injectable solutions at different pH, varying temperatures and ascended time intervals [26,27]. Upon stress acid hydrolysis for KZ and FZ, they were found to be almost stable.

In the present work, the two described chromatographic methods were successfully applied for the assay of the studied drugs in pharmaceutical preparations.

2. Experimental

2.1. Apparatus

High-performance liquid chromatograph, composed of a dual pump (LC-10 AD with an ultraviolet variable wavelength detector SPD-6 AV (Shimadzu, Kyoto, Japan). A Rheodyne injector model 7161 (California, USA), equipped with 20-μl injector loop, and an integrator model C-R7A (Shimadzu).

TLC Scanner; CS-9301 PC. Dual wavelength flying spot scanning densitometer (Shimadzu). Precoated TLC-plates, silica gel 60 F_{254} (20 × 20 cm, 0.25 mm), (E. Merck, Darmstadt, FRG). Hamilton analytical syringes, 10 and 25 μl (Nevada, USA).

2.2. Materials

2.2.1. Reference materials

Clotrimazole was kindly supplied by Alexandria Pharm. Co. Alex., Egypt, its purity as assayed by B.P. 1998 [28], was found to be 99.79 ± 0.38% ($n = 5$).

Ketoconazole was kindly obtained from Janssen Pharmaceutica, Beerse, Belgium, its assay way found to be 99.43 ± 0.31% ($n = 5$) according to the USP 24 (NF 19, 2000) [29].

Fluconazole was kindly obtained from Pfizer-Egypt, (99.99 ± 0.53%, $n = 5$) purity according to the manufacturer recommended spectrophometric method.

Imidazole was purchased from Sigma Chemical Company, MO, USA (100.01% reported purity).

2.2.2. Pharmaceutical formulations

Cansten® cream (batch number 7436071), Cansten® vaginal tablets (batch number 7438015) and Cansten® topical solution (batch number 7437036), labeled to contain 10 mg/g, 100 mg per tablet and 10 mg/ml of clotrimazole, respectively (Alex. Co.).

Nizoral™ cream (batch number 96K281090) and Nizoral™ tablets (batch number 981031610), labeled to contain 20 mg/g and 200 mg per tablet of ketoconazole, respectively (Janssen Pharmaceutica, Beerse, Belgium).

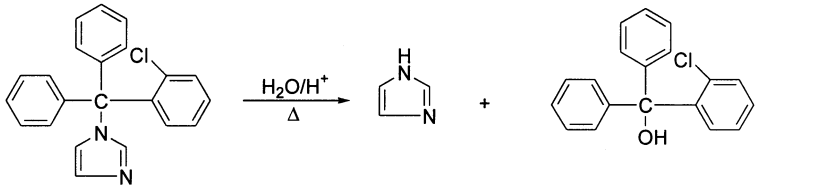
Diflucan capsules (50 mg fluconazole per capsule, batch number 7201, 7210 and 150 mg fluconazole per capsule batch number 7101, 62592108) and diflucan intravenous infusion (I.V.) labeled to contain 2 mg fluconazole per 1 ml solution, batch number 72592702 and 72592703, (Pfizer, Egypt).

2.2.3. Chemicals and reagents

Methanol, chloroform and acetone (Adwic, El-Nasr Pharm. Co., Cairo, Egypt). Acetonitril and methanol (HiPerSolv®, HPLCgrade, E. Merck, Darmstadt, FRG). All other chemicals were of analytical grade.

2.2.4. Standard solutions for linearity studies

1. Standard stock solutions of 1 mg/ml CZ were prepared in methanol for TLC–densitometry, and in the mobile phase for HPLC.



Clotrimazole Imidazole (o-chlorophenyl)diphenyl-methanol

Scheme 1. Acid-degradation of clotrimazole.

2. Standard stock solutions of 1 mg/ml KZ and 0.5 mg/ml FZ were prepared in mobile phase for HPLC.

2.3. Analytical techniques

2.3.1. HPLC method

2.3.1.1. Chromatographic conditions. Chromatographic separation was carried out at ambient temperature on a μ-Bondapak™ C_{18} [(25 cm × 4.6 mm, i.d.), HPLC cartidge columnn, Waters Corp., MA, USA]. The compounds were separated isocratically with a mobile phase consisting of a mixture of acetonitrile + 25 mM trishydroxymethyl aminomethane in phosphate buffer (pH 7) = 55:45, v/v). The pH of the binary solvent mixture was finally adjusted to 7.0 with *o*-phosphoric acid. The mobile phase was filtered through 0.45 μm Millipore membrane filters and degassed for ~15 min in an ultrasonic bath prior to use. To reach equilibrium, the analysis was usually started after the passage of 50–60 ml of mobile phase. The flow rate was 2 ml/min for CZ-and KZ-elutions, while, FZ was eluted at 1.5 ml/min. The injection volume was 20 μl, and the eluted analytes for the three drugs were traced by UV-detection at 260 nm, with 0.001 AUFS sensitivity.

2.3.1.2. Construction of calibration curves. Prepare three separate sets, each consisting of six 10 ml volumetric flasks. In the first set, transfer different volumes of CZ-stock solution (0.2–7.5 ml), followed by 1 ml of KZ-stock solution as (IS). In the second set, different aliquots portions of KZ-stock solution (0.8–8 ml) were transferred and mixed with 1 ml of CZ-stock solution as IS. In the third set, transfer volumes of 0.5–5 ml from FZ-stock solution followed by 1 ml KZ-stock solution as IS. Dilute the contents of all flasks in the three sets with the mobile phase. Triplicate 20 μl injections were made for each solution and the peak area ratios of each drug to its internal standard were plotted against the corresponding concentrations to obtain the calibration graphs for the three studied drugs. The regression equations were computed for each one.

2.3.1.3. Samples preparation and procedure.

A) Clotrimazole

a) Acid degraded standard solution of CZ (equivalent to 1 mg/ml of degraded CZ): transfer 100 mg of pure clotrimazole to a 100-ml conical flask. Add 20 ml 5 N HCl, reflux on a boiling water bath for 2 h, evaporate to a concentrated volume under vacuum. Cool to room temperature (r.t.) and dilute to volume with methanol. Complete acid degradation of CZ was confirmed by TLC fractionation, where 20 μl of pure (1 mg/ml), and degraded CZ-solution were applied separately on 10 × 10 cm TLC plate. The spots were separated using chloroform + acetone + 25% aqueous ammonia (7:1:0.1, by volumes) as a mobile phase and visualized by iodine vapor. No spot corresponding to intact CZ was detected from the degraded sample.

b) Laboratory prepared mixtures: transfer accurately aliquot portions (1–9 ml) of the intact CZ-standard solution (500 μg/ml) in a series of 10 ml measuring flasks. Add aliquot portions of the degraded CZ-sample (500 μg/ml) to prepare different mixtures containing 10–90% of the degradation products and complete to volume with methanol. The mixtures were chromatographed using the specified chromatographic procedure and the concentrations of the intact drug were calculated from the corresponding regression equation (Table 1).

c) Dosage formulations:
- cream (Cansten®): a portion of cream containing ~10 mg CZ was accurately weighed in a 100 ml beaker, 5 ml of KZ stock solution (IS) was added. Extract with two 20 ml aliquots of mobile phase by warning in a 60 °C water bath with occasional shaking. The beaker was then removed from the bath, shaked vigorously again for 5 min, cooled in ice for 15 min, and filtered through filter paper into a 50 ml volumetric flask. The volume was completed with the same extracting solvent;
- vaginal tablets (Cansten®): transfer a weight of finely powdered 20 tablets equivalent to ~10 mg CZ into a 100 ml beaker, followed by 5 ml of KZ (IS). Extract with 20 ml aliquots of mobile phase by vigorous mechanical shaking for 2 min, followed by filtration into a 50 ml measuring flask. Dilute to volume with the same extracting solvent;
- solution (Cansten®): a volume of sample equivalent to ~10 mg CZ was accurately transferred to a 50 ml measuring flask, followed by 5 ml KZ as IS. Dilute to volume with mobile phase.

Table 1
Percentage recovery of pure clotrimazole in presence of degradation products by the two suggested methods

HPLC-method		TLC-method	
Degradation (%)	Recovery of CZ (%) [a]	Degradation (%)	Recovery of CZ (%) [a]
10	100.01	20	99.75
30	100.36	40	100.53
50	99.90	50	99.87
70	99.93	60	99.50
90	100.02	80	100.80
Mean ± CV	100.08 ± 0.20	Mean ± CV	100.09 ± 0.55

[a] Average of three experiments.

B) Ketoconazole

Cream and tablets (Nizoral™): samples were prepared as described under Cansten® cream and Cansten® tablets, respectively, using CZ as IS.

C) Fluconazole

- capsules (Diflucan): the contents of ten capsules were mixed well and a weight of powder equivalent to 5 mg FZ was accurately transferred to a small beaker. The procedure was completed as described under Cansten® tablets, starting from 'followed by 5 ml of KZ....';
- IV. Infusion Diflucan: a volume of sample equivalent to 5 mg FZ was accurately transferred into a 50 ml measuring flask, followed 5 ml KZ. The volume was completed with mobile phase;
- all previous prepared dosage form samples were finally filtered through 0.45 μm membrane filters and 20 μl aliquot of samples were chromatographed using the specified chromatographic parameters described under construction of calibration curves. Apply the standard addition technique to the analysis of the dosage forms and calculate the percentage recoveries of pure drugs using the corresponding regression equation.

2.3.2. TLC–densitometry

2.3.2.1. Construction of calibration curve of clotrimazole. Different aliquots (5–25 μl) of clotrimazole standard solution (1 mg/ml) were applied to a TLC plate (20 × 20 cm) using 25 μl Hamilton syringe. Spots are located 2 cm apart from each other and 2 cm from the bottom edge of the plate. Develop the air-dried plate in a chromatographic tank, previously saturated for at least 1 h with the developing mobile phase, chloroform + acetone + 25% aqueous ammonia (7:1:0.1, by volumes), by ascending chromatography through a distance of 15 cm at r.t. The plate was then scanned for CZ at 260 nm. The area under the peaks (AUP) were recorded and plotted against the corresponding concentration to get the calibration graph.

2.3.2.2. Samples preparation and procedure.

a) Acid degraded standard solution of CZ: prepared as mentioned under HPLC method.
b) Laboratory prepared mixtures: aliquot portions (2–8 ml) of the intact CZ standard solution (1000 μg/ml) were transferred accurately in series of 10 ml measuring flasks. Add aliquot portions of the degraded CZ sample (1000 μg/ml) were added to prepare different mixtures containing 20–80% of the degradation products and completed to volume with methanol. Portions (25 μl) of the prepared mixtures were applied on 10 × 10 cm TLC-plate. The chromatographic assay described earlier was followed.
c) Pharmaceutical formulations:
   - cream (Cansten®): a quantity of cream equivalent to 20 mg CZ was transferred to a 100 ml beaker. The procedure was completed as mentioned under sample preparations of Cansten® cream for HPLC method starting from 'Extract with two 20 ml. . ..';
   - vaginal tablets (Cansten®): a weight of finally powdered tablets equivalent to 20 mg CZ was transferred into a small beaker. The extraction method was followed as described under sample preparation of vaginal tablets of HPLC method starting from 'Extract with two 20ml.....';
   - solution (Cansten®): an aliquot volume equivalent to 20 mg CZ was diluted to 50 ml with methanol in a 50-ml volumetric flask.

Of the previously prepared samples, 25 μl were applied separately on (10 × 10 cm) TLC-plates. The chromatographic assay described above was followed. The accuracy of the method was assessed by applying the standard addition technique to the analysis of the pharmaceutical formulations and the mean percentage recoveries of pure drugs were calculated from the corresponding regression equation.

3. Results and discussion

The purpose of this study is to investigate the feasibility of utilizing simple and selective chromatographic methods for the determination of the antifungal drugs, clotrimazole, ketoconazole and fluconazole in pure forms and in pharmaceutical preparations. Also the suggested HPLC and TLC-densitometric methods were utilized as stability indicated methods for estimation of clotrimazole in presence of its acid degradation products.

3.1. HPLC method

The method involves the use of μ-Bondapak™ C_{18} (25 cm × 4.6 mm, i.d.) and a mobile phase consisting of acetonitrile + 25 mM trishydroxy methyl aminomethane in phosphate buffer (pH 7), (55:45, v/v). The mobile phase was chosen after several trials with different solvents in various proportions and different pH values. The chromatographic system described allows complete base line separation of clotrimazole from its degradation products with approximate retention times of 9.2, 3.4 and 2.0 min for clotrimazole, (*o*-chlrophenyl)-diphenyl methanol and imidazole, respectively.

The imidazole fraction which has a retention time of about 2 min in this system, has virtually only weak UV-

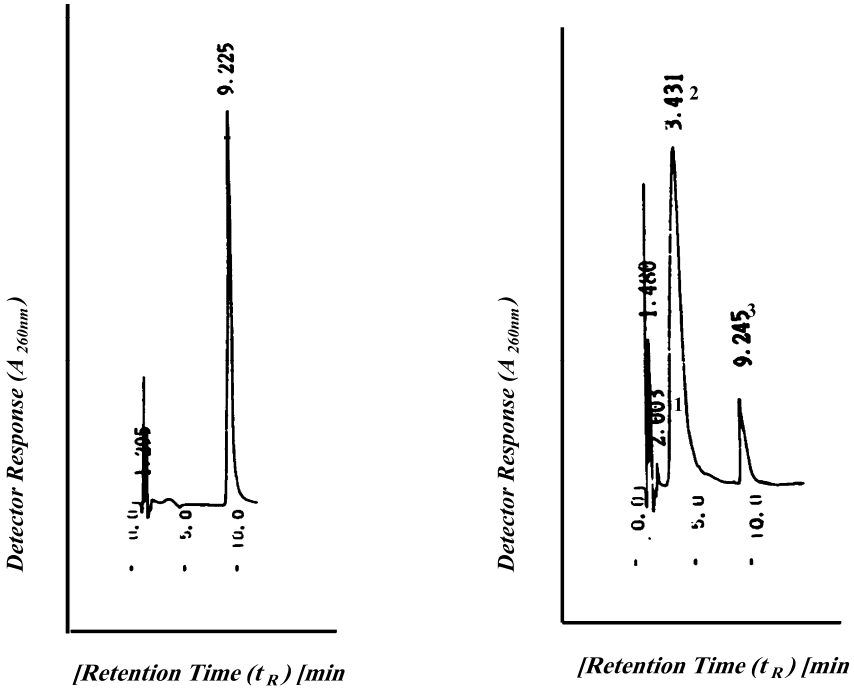


Fig. 1. HPL chromatogram of pure (a) and degraded (b) clotrimazole (100 μg.ml^{-1}); 1 = imidazole; 2 = (o-chlorophenyl) diphenyl methanol; 3 = clotrimazole.

absorptivity at 260 nm, (Fig. 1a, b). It was found that the detector response was linear in concentration range of 20–750 μg/ml for clotrimazole when plotted against the corresponding peak area ratios to internal standard. The mean recoveries of pure clotrimazole samples were found to be 99.83 ± 0.40% ($n = 7$), using the following regression equation:

$$A = 0.0062 \times C + 0.0020, \quad (r = 0.9999)$$

where A is the peak area ratios, C the concentration (μg/ml) and r is the correlation coefficient.

The statistical evaluation of the regression lines [30,31] was done by calculating the standard deviations of residues ($S_{x/y}$), of the intercept (S_a), and of the slopes (S_h), where, the small figures (Table 1) indicate a high precision of the proposed methods even in the presence of the drug degradates.

The suggested method could be applied as stability indicating method for the determination of clotrimazole in laboratory prepared mixtures containing up to 90% of its acid degradation products with mean percentage recovery of 100.08 ± 0.20%. Table 1 shows the results obtained.

The same method was successfully applied for simple and rapid estimation of ketoconazole and fluconazole in pure forms and in pharmaceutical preparations. KZ was eluted at a flow rate of 2 ml/min with retention time of ~5.7 min (Fig. 2), while, FZ at the same flow rate was eluted too fast almost close to the solvent side. The flow rate was adjusted then at 1.5 ml/min to separate FZ at a reasonable time of ~2.4 min for maximum detector response, away from the peak of solvent (Fig. 3). Limit of detection (LOD), determined by signal to noise ratio ($S/N = 2$), for CZ, KZ and FZ were determined to be 10

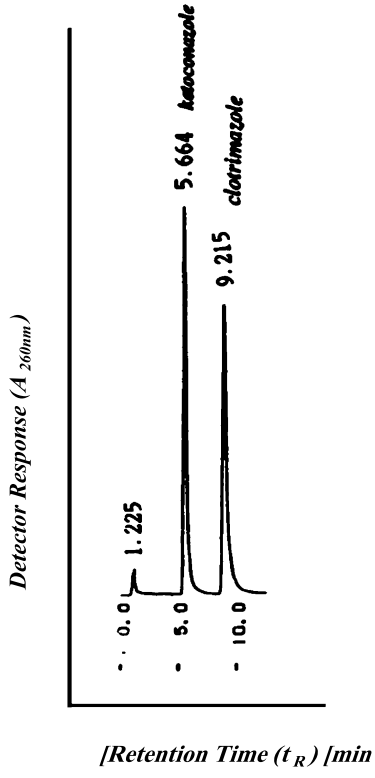


Fig. 2. A typical HPL chromatogram of resolved ketoconazo and coltrimazole.

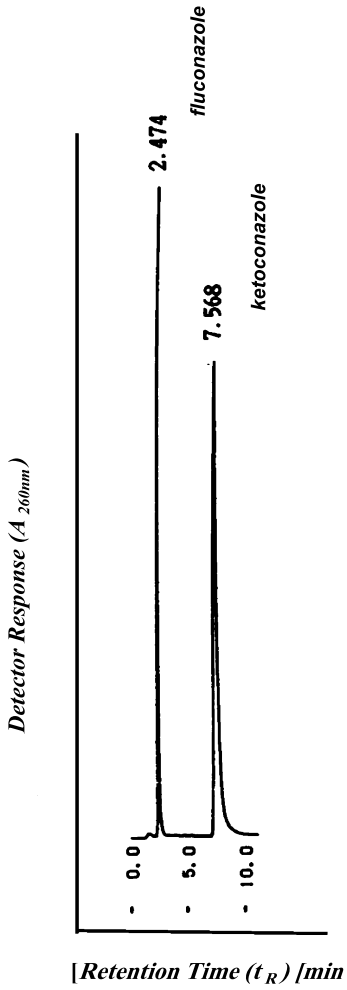


Fig. 3. A typical HPL chromatogram of resolved, fluconazole and ketoconazole (IS).

μg/ml (2.89×10^{-5} mol/l), 10 μg/ml (1.88×10^{-5} mol/l) and 5 μg/ml (1.63×10^{-5} mol/1), respectively.

Table 3
Application of standard technique to the analysis of clotrimazole in pharmaceutical preparations using the proposed TLC method

Pharmaceutical preparations	Assay, recovery ± CV [a]	Standard addition, recovery ± CV [a]
Cansten® cream batch number 7436071	100.16 ± 0.77	100.89 ± 0.31
Cansten® tablets batch number 7438015	100.06 ± 0.43	100.33 ± 0.45
Cansten® solution batch number 7437036	100.22 ± 0.30	100.94 ± 0.62

[a] Average of five experiments.

The linearity of detector response against the peak area ratios was calculated to be 80–800 and 20–250 μg/ml, with mean recoveries of 99.93 ± 0.56% ($n = 6$) and 100.02 ± 0.24% ($n = 6$) for KZ and FZ, respectively. The regression equations were found to be:

KZ: $A = 0.0070 \times C - 0.0282$, $(r = 0.9999)$

FZ: $A = 0.0197 \times C + 0.0303$, $(r = 0.9999)$

The validity of the proposed method was assessed by the application of standard addition technique to the analysis of pharmaceutical formulations. The results obtained are listed in (Table 2).

3.2. TLC-densitometric method

Different developing systems were tried among which complete separation of clotrimazole and its related substances was achieved using chloroform + acetone + 25% ammonia (7:1:0.1, by volumes). The R_f values of clotrimazole, (2-chlorophenyl)-diphenyl methanol and imidazole were found to be 0.75, 0.94, 0.02, respectively. Imidazole being UV-non absorbing substance at 260 nm, it can be visualized only by iodine vapors. The

Table 2
Application of the HPLC method to the pharmaceutical preparations and validation of the method by standard addition technique

Pharmaceutical preparations	Assay, recovery ± CV	Standard addition, recovery ± CV [a]
(I) *Clotrimazole preparations*		
Cansten® cream batch number 7436071	101.15 ± 0.38 ($n = 5$)	99.98 ± 0.59
Cansten® vaginal tablets (0.l gm per tablet) batch number 7438015	99.35 ± 0.32 ($n = 5$)	99.51 ± 0.39
Cansten® solution batch number 7437036	100.29 ± 0.46 ($n = 5$)	100.69 ± 0.25
(II) *Ketoconazole preparations*		
Nizoral™ tablets batch number 981031610	102.21 ± 0.50 ($n = 5$)	101.46 ± 0.50
Nizoral™ cream batch number 96K281090	101.39 ± 0.47 ($n = 5$)	100.15 ± 0.75
(III) *Fluconazole preparations*		
Diflucan 50 mg capsules batch number 7201	100.67 ± 0.36 ($n = 6$)	100.16 ± 0.47
Diflucan 50 mg capsules batch number 7210	100.38 ± 0.18 ($n = 6$)	99.90 ± 0.45
Diflucan 150 mg capsules batch number 7101	101.59 ± 0.40 ($n = 6$)	100.45 ± 0.28
Diflucan 150 mg capsules batch number 62592108	100.42 ± 0.48 ($n = 6$)	99 97 ± 0.14
Diflucan I.V. infusion batch number 72592703	100.03 ± 0.13 ($n = 6$)	100.37 ± 0.34
Diflucan I.V. infusion batch number 72592702	100.03 ± 0.19 ($n = 6$)	100.18 ± 0.33

[a] Average of five experiments.

Table 4
Statistical comparison between the results obtained by the proposed methods and reference ones for the analysis of the studied drugs in pure form

Values	Clotrimazole			Ketoconazole		Fluconazole	
	HPLC method	TLC method	Official method BP-1998[28]	HPLC method	Official method USP-24[29]	HPLC method	Reference method [b]
Mean	99.83	99.55	99.79	99.93	99.43	100.02	99.99
SD	0.40	0.74	0.38	0.56	0.31	0.23	0.53
n	7	9	5	6	5	6	6
Variance	0.157	0.550	0.145	0.309	0.098	0.057	0.283
Student's *t*	0.109 (2.228) [a]	0.546 (2.179) [a]		1.783 (2.262) [a]		0.134 (2.228) [a]	
F	1.080 (6.16) [a]	3.80 (3.84) [a]		3.153 (6.26) [a]		4.96 (5.05) [a]	

[a] The values in the parenthesis are the corresponding theoretical values of t and F at $\alpha = 0.05$.
[b] Reference method is the method adopted for fluconazole by Pfizer, Egypt.

difference of the R_f values permit the quantitative estimation of clotrimazole by scanning densitometry at 260 nm. LOD for clotrimazole was found to be 2.5 μg per spot (7.2×10^{-6} μg per spot). A linear correlation was obtained between the peak area and the concentration range of 5–25 μg per spot of CZ with mean accuracies of $99.55 \pm 0.74\%$ ($n = 9$) using the following polynomial regression equation:

$$A = 0.1156C^2 + 2.9605C + 0.0291, \quad (r = 0.9999)$$

where A is the peak area, C the concentration (μg per spot) and r is the correlation coefficient.

The proposed method was successfully applied as a stability indicating method for the determination of clotrimazole in laboratory prepared mixtures containing up to 80% of its acid-degradation products with mean percentage recovery of $100.09 \pm 0.55\%$ (Table 1).

The accuracy of the method was validated by applying the standard addition technique to the analysis of pharmaceutical preparations. The results are listed in (Table 3). The results obtained by both suggested methods were evaluated and compared with those obtained by the official and reference methods (Table 4).

4. Conclusions

Beside a sensitive HPLC-method, the present work gives another analytical application, making use of coupling TLC-fractionation with densitometry, for the selective quantification of the named azolesantifungal agents, singly and if admixed, and also in the presence of some of their byproducts and acid-degradates. From the previous results, it has been seen that the two methods are sufficiently accurate and quite reproducible for the selective determination of the studied drugs.

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