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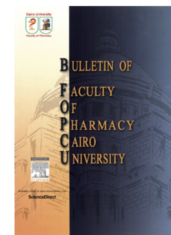

ORIGINAL ARTICLE

HPLC and TLC chromatographic methods for simultaneous determination of binary mixture of isoconazole nitrate and diflucortolone valerate

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KEYWORDS

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Abstract HPLC and TLC-densitometric methods were used to determine a binary mixture of isoconazole (ISO) and diflucortolone (DIF). For HPLC method a rapid separation could be achieved on a C18 column using mobile phase of 80% acetonitrile–20% methanol. The components were monitored at 230 nm over a concentration range of 10–90 μg mL^{-1} for ISO and 2–18 μg mL^{-1} for DIF with mean percentage recoveries 99.95 ± 0.866 and 99.98 ± 0.744, respectively. The second method is TLC-densitometric, where ISO and DIF are separated on silica gel plates using ethyl acetate: chloroform: toluene (60:10:10 by volume) as a developing system and scanning of the separated bands at 215 nm over a concentration range of 0.1–4 μg $spot^{-1}$ for ISO and scanning of the separated bands at 237 nm over a concentration range of 0.1–1.4 μg $spot^{-1}$ for DIF with mean percentage recoveries 100.19 ± 0.956 and 100.1 ± 0.689 for ISO and DIF, respectively. The suggested methods were used to determine both drugs binary mixture in pure form and dosage form. The validity of the proposed methods was further assessed by applying standard addition technique. The obtained results were statistically compared with official HPLC method, showing no significant difference with respect to accuracy and precision.
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1. Introduction

Isoconazole nitrate (ISO) (Fig. 1a), is a broad-spectrum imidazole derivative topical antifungal drug. It is chemically designated as 1-[(2RS)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole. It is used for the treatment of Candida species and dermatophytes.[1] Different HPLC methods have been reported for the determination of isocnazole.[2–4] Diflucortolone valerate (Fig. 1b), 6α,9α-difluoro-3,20-dioxo-11β-hydroxy-16α-methylpregna-1,4-dien-21-yl valerate is an anti-inflammatory corticosteroid.[1] A HPLC method for determination of diflucortolone valerate is reported in literature.[5] Binary mixture of these drugs is used for the treatment of eczematous mycoses.[6] Only two methods were reported for the simultaneous determination of these components in

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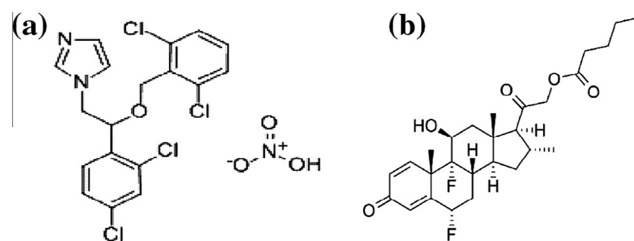

Figure 1 Structures of isoconazole nitrate (a) and diflucortolone valerate (b).

their pharmaceutical formulations as HPLC and spectrophotometry.[7,8]

Both HPLC methods utilized different mobile phases which consist of methanol–water (95:5 v/v) on Phenomenex ODS (250 × 4.6 mm; 5 μm) for HPLC and methanol–water (69:31, v/v) for UPLC on Acquity HSS C18 (50 × 2.1 mm; 1.8 μm).

2. Experimental

2.1. Instruments

1. HPLC instrument
   – Agilent 1100 series, equipped with a variable wavelength detector and 20 μL volume injection loop using Column of ODS C18-Agilant HC.C18 (2)-5 μm (4.6 × 250 mm I.D.), U.S.A.

2. TLC-densitometric instrument
   – Camag TLC scanner 3 S/N 130319 operated with winCATS software.
   – Linomat IV with 100 μL syringe (Camag, Muttenz, Switzerland).
   The following requirements are taken into consideration:
   i. Slit dimensions: 6 × 0.3 mm.
   ii. Scanning speed: 20 mm s^{-1}.
   iii. Spraying rate: 10 s μL^{-1}.
   iv. Data resolution: 100 μm $step^{-1}$.
   v. Band width: 5 mm.
   vi. Result output: Chromatogram and integrated peak area.

3. TLC plates precoated with silica gel 60 F254 (20 × 20), 0.2 mm thickness (E. Merck), Darmstadt, Germany.
4. UV short wavelength (254 nm) lamp. (Desaga, Germany).
5. Ultrasonic bath, Soniclean pty. Ltd 160T, HF-Frequency 50/60 Hz (10 L capacity), Australia.

2.2. Reagents and solvents

All reagents used throughout this work were of analytical pure grade, and solvents were of HPLC grade: methanol, acetonitrile, chloroform, ethyl acetate and toluene (HPLC grade) (Merck).

2.3. Samples

– Pure samples were kindly supplied by Misr Company for pharmaceutical industries. The percentage purity was found to be 99.56 ± 0.649% and 99.96 ± 0.767 for ISO and DIF, respectively according to the official method.[1]
– Travodermal cream, Batch No. 906023, manufactured by Misr Company for pharmaceutical industries and were purchased from a local market.

2.4. Standard stock and working solutions

A stock standard solution of ISO and DIF (1 mg/mL) were prepared by dissolving separately ISO and DIF in methanol then completing to 100 mL measuring flask with the same solvent. Aliquot of the prepared stock solution was further diluted with methanol to get working solution with final concentration (100 μg mL^{-1}).

2.5. Procedures

For HPLC method:

Chromatographic conditions:

Chromatographic separation was carried out using isocratic mode on a Agilant ODS-C18 (4.6 × 250 mm I.D.) column with a mobile phase consisting of 80% acetonitrile: 20% methanol. (The mobile phase was filtered using 0.45 m membrane filter and degassed by ultrasonic vibrations for 10 min) with a flow rate of 1 mL/min and the eluate was scanned at 230 nm at room temperature. All the injections were run in three replicates and the injection volume was 20 μL. The run time was 6 min and the total peak areas were used to quantify the studied components.

2.5.1. Construction of calibration graph for the determination of the binary mixture by HPLC method

Aliquots of ISO and DIF working standard solution (100 μg mL^{-1}) equivalent to 100–900 μg and 20–180 μg, respectively were accurately transferred into a two series of 10-mL volumetric flasks, the volume was completed to the mark with the mobile phase. Analyze the prepared samples using the previously mentioned chromatographic conditions and record the peak area then construct a calibration curve correlating the peak areas of ISO and DIF to the corresponding concentrations. Compute the corresponding regression equations.

2.5.2. Construction of calibration graph for the determination of ISO and DIF by TLC-densitometric method

Aliquots equivalent to 0.05–2 mg, 0.05–0.7 mg of ISO and DIF, respectively from stock standard solution (1 mg mL^{-1}) were transferred into 10-mL volumetric flasks and the volume was completed with methanol. 20 μL was applied to thin layer chromatographic plates (20 × 20) using applicator. Spots were spaced 2 cm apart from each other and 1.5 cm from the bottom edge of the plate. The plates were developed in the chromatographic tank previously saturated with the developing mobile phase, ethyl acetate: chloroform: toluene (60:10:10 by volume), for at least 20 min. The plates were developed by

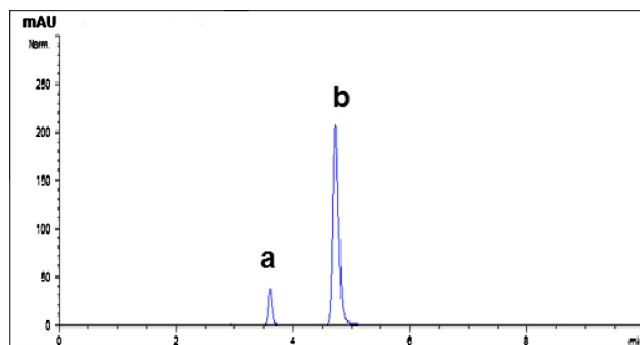

Figure 2 HPLC chromatogram of (a) DIF (4 μg/mL), (b) ISO (40 μg/mL).

Table 1 Parameters required for the system suitability test of the HPLC method.

Parameters	HPLC method		Densitometric method	
	ISO	DIF	ISO	DIF
Resolution	6.3		4.5	
T (tailing factor)	1.16	1	0.57	1.05
α (selectivity factor)	1.3		23.7	
K′ (column capacity)	22.5	17	4.5	0.19
N (column efficiency)	7478	12,189	16.6	815.7
HETP = *L* (length of column in cm)/*N*	0.003	0.002		

ascending technique to a distance of about 8 cm, dried at room temperature. The spots were detected under UV-lamp and scanned at 215 nm and 237 nm for ISO and DIF, respectively. The peak areas were recorded for the drug. The calibration curves were constructed by plotting the area under the peak versus the corresponding concentrations. The corresponding regression equations were computed.

2.5.3. Determination of the binary mixture in pharmaceutical dosage form by the proposed methods

One gram of cream was extracted by 35 mL methanol, heated to 50 °C till it melted and sonicated for ten minutes. The extracted sample was cooled for 2 h till base coalescence. The extract was filtered into a 50 mL volumetric flask and the residual mass was re-extracted by 10 mL methanol, cooled and filtered then the volume was completed to 50 mL with the same solvent.

The proposed methods were applied for the analysis of the pharmaceutical preparation solutions using the procedures mentioned under linearity and construction of calibration curve for each method and the concentrations of the cited drugs were calculated from the corresponding regression equations.

3. Results and discussion

This work is concerned with the simultaneous determination of ISO and DIF using two different chromatographic techniques. First method is HPLC which represents an advantage over the previously published HPLC method[7,8] in sensitivity. Different mobile phases were tried for the chromatographic separation of the components and the best resolution was achieved using a mobile phase composed of 80% acetonitrile–20% methanol. Using the specific chromatographic conditions retention times were found to be 4.7 and 3.6 for ISO and DIF, respectively Fig. 2. Statistical analysis of the parameters required for system suitability test of HPLC method indicates a good resolution of the two components as in Table 1.

HPLC method allows selective determination of ISO and DIF in the range of 10–90 μg mL^{-1} and 2–18 μg mL^{-1}, respectively. The regression equations were calculated and found to be:

$$A = 34.703C + 36.745 \quad r = 0.9999 \quad \text{for ISO at 230 nm}$$

$$A = 42.689C + 20.1 \quad r = 0.9999 \quad \text{for DIF at 230 nm}$$

where, "*A*" is the peak area. "*C*" is the concentration in μg mL^{-1} and "*r*" is the regression coefficient. The mean percentage recoveries and standard deviations of the pure drug were calculated as shown in Table 2.

The second method is a TLC-densitometric procedure, different systems were tried, where complete separation of ISO and DIF was achieved using ethyl acetate: chloroform: toluene (60:10:10 by volume) as the mobile phase. The R_f values were 0.18 for ISO and 0.84 for DIF as in Figs. 3 and 4.

TLC-Densitometric technique allows selective determination of ISO and DIF in the range of 0.1–4 μg spot^{-1} for ISO and 0.1–1.4 μg spot^{-1} for DIF. The regression equations were calculated and found to be:

Table 2 Regression parameters and results of determination of pure samples of ISO and DIF by the proposed methods.

Parameters	HPLC method		Densitometric method	
	ISO	DIF	ISO	DIF
Linearity (μg/mL)	10–90	2–18	0.1–4	0.1–1.4
Slope	34.703	42.689	−298.38	6248.2
Intercept	36.745	20.12	581.66	298.87
Correlation coefficient (*r*)	0.9999	0.9999	0.9999	1
Mean ± SD	99.95 ± 0.866	99.98 ± 0.744	100.19 ± 0.956	100.10 ± 0.689
Accuracy	100.13 ± 0.984	100.29 ± 0.748	100.15 ± 1.528	100.57 ± 1.340
Precision				
Repeatability (intraday)	1.315	1.521	1.827	0.952
Intermediate precision (interday)	1.209	0.864	1.200	1.157

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Table 3 Determination of studied drugs in cream by the proposed methods.

	HPLC method		Densitometric method	
	ISO[a]	DIF[a]	ISO[a]	DIF[a]
Travodermal cream Batch NO. 906023[b]	97.40 ± 0.90	97.90 ± 0.75	97.99 ± 1.97	100.36 ± 0.86
Standard addition[b]	99.97 ± 1.45	99.37 ± 1.33	99.61 ± 1.58	99.56 ± 0.81

[a] Recovery ± RSD%.
[b] Average of at 3 experiments.

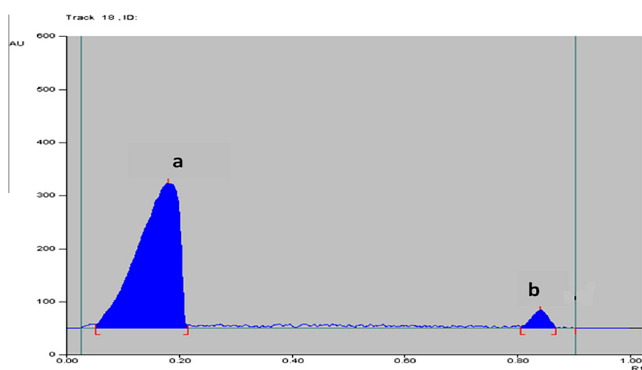

Figure 3 TLC-densitometric chromatogram of (a) ISO (4 μg/band), (b) DIF (0.4 μg/band) at 215 nm.

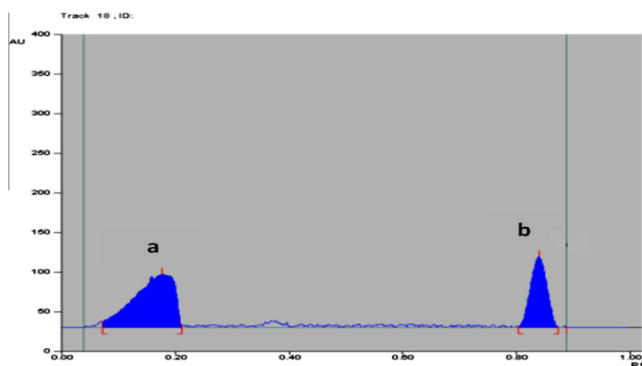

Figure 4 TLC-densitometric chromatogram of (a) ISO (4 μg/band), (b) DIF (0.4 μg/band) at 237 nm.

$$A = -298.38C^2 + 5131.6C + 581.66$$
$$r = 0.9999 \quad \text{for ISO at 215 nm}$$

$$A = 6248.2C + 298.87 \quad r = 1 \quad \text{for DIF at 237 nm}$$

where, "A" is the peak area. "C" is the concentration in μg spot^{-1} and "r" is the regression coefficient.

4. Methods validation

Method validation was performed according to ICH guideline[9] for the proposed methods as follows:

4.1. Range and linearity

The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. The calibration curves were constructed within concentration ranges that were selected on the basis of the anticipated drug concentration during the assay of the dosage form. Each concentration was repeated three times. The linear regression equations are summarized in Table 2. The corresponding concentration ranges, calibration equations and other statistical parameters for all methods are listed in Table 2.

4.2. Accuracy

To study the accuracy of the proposed methods, procedures under linearity for the drugs were repeated three times for the determination of six different concentrations of pure MIC, MF, and GEN. The accuracy expressed as percentage recoveries is shown in Table 2. The standard addition technique presented in Table 3 showed no interference of

Table 4 Statistical analysis of the proposed methods and the official methods of isoconazole (ISO) and diflucortolone (DIF) in their pure form.

	HPLC method		Densitometric method		Official method	
	ISO	DIF	ISO	DIF	DIF*	ISO**
Mean	99.95	99.98	100.19	100.10	99.96	99.56
SD	0.866	0.744	0.956	0.689	0.767	0.649
n	9	9	8	8	9	6
Variance	0.75	0.554	0.914	0.475	0.589	0.421
Student t-test	0.944 (2.16)	0.062 (2.12)	1.393 (2.16)	0.388 (2.131)		
F	1.781 (4.82)	1.06 (3.44)	2.171 (4.82)	1.240 (3.73)		

Figures in parenthesis are the corresponding theoretical values at $P = 0.05$.
* HPLC using C_{18} column and mobile phase of 25:75 water: acetonitrile at 238 nm.
** Non aqueous titration, end point was determined potentiometrically.

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pharmaceutical excipients. Good accuracy of the developed methods was indicated by the obtained results.

4.3. Precision

The intra-day and inter-day precisions of the proposed methods were determined by the analysis of three different concentration of the proposed drugs, within the linearity range, by three replicate analysis of three pure samples of the drugs on a single day and on three consecutive days, the results are illustrated in Table 2.

4.4. Selectivity

Selectivity was ascertained by analyzing different mixtures containing the drugs in different ratios within the linearity range. The RSD showed good percentage recoveries with the lowest standard deviation among the other methods. Satisfactory results are shown in Table 2.

The proposed methods were also applied for the determination of ISO and DIF in its dosage form. Furthermore, the validity of the methods were assessed by applying the standard addition technique, as in Table 3, mean percentage recovery revealed that there was no interference from any excipients, that may be found in the pharmaceutical dosage forms. The results obtained by applying the proposed methods were statistically compared with the official method[1] and no significant difference was found regarding accuracy and precision as in Table 4.

5. Conclusion

The presented HPLC and TLC-densitometric methods could provide highly selective and quantitative methods for the simultaneous analysis of ISO and DIF, which represent an advantage over the previously published methods. The presented isocratic HPLC method could provide highly sensitive method for the determination of ISO and DIF at single wavelength in short analysis time.

The TLC-densitometric method has the advantage of short run time, large sample capacity and the use of minimal volume of solvent. The results obtained indicate that the proposed methods can be classified among highly sensitive methods. These merits suggest the use of the proposed method in routine and quality control analysis without the interference of commonly encountered dosage form additives.

Conflict of interest

The authors declare no conflict of interest.

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