

METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CLOTRIMAZOLE, MICONAZOLE NITRATE, AND TINIDAZOLE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD IN TABLETS

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ABSTRACT

Objective: The aim of the study is to develop and validate a high-performance liquid chromatographic method for the simultaneous determination of clotrimazole, miconazole nitrate, and tinidazole tablet dosage forms.

Materials and Methods: A Waters C₁₈ column (50 mm×4.6 mm, 5 μm) with mobile phase consisting of acetonitrile, methanol, and water 55:25:20 (v/v) (pH 2.5 adjusting with 0.5% orthophosphoric acid) was used. The flow rate was 1.0 ml/min, and effluents were monitored at 210 nm.

Results: The retention time of miconazole nitrate, tinidazole, and clotrimazole tablets was found to be 2.9 min, 3.5 min, and 4.7 min, respectively. The method was validated according to the ICH guidelines for specificity, limit of detection, limit of quantification, precision, accuracy, linearity, ruggedness, and robustness.

Conclusion: The method shows good reproducibility and recovery with % relative standard deviation <2. Hence, the proposed method was found to be simple, specific, precise, accurate, and linear. Hence, it can be applied for routine analysis of clotrimazole, miconazole nitrate, and tinidazole in pharmaceutical combined dosage forms.

Keywords: Reversed-phase high-performance liquid chromatography, Clotrimazole, Miconazole nitrate, Tinidazole, ICH guidelines.

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INTRODUCTION

Nowadays, combined dosage forms are mostly preferred when compared to single dosage forms. Clotrimazole, miconazole nitrate, and tinidazole are available in combined pharmaceutical dosage form. The brand name is Defung vaginal tablet containing 20 mg of clotrimazole, 100 mg of miconazole nitrate, and 500 mg of tinidazole for the treatment of various vaginal infections [1]. Literature survey reveals that various analytical methods have been reported for single dosage forms. The present paper aims to report a simple, accurate, precise, and reversed-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous estimation of clotrimazole, miconazole nitrate, and tinidazole in combined dosage forms.

Drug profile of clotrimazole

Its IUPAC name is 1-[(2-chlorophenyl)-diphenylmethyl]imidazole (Fig. 1). The molecular formula is C₂₂H₁₇ClN₂. It is an antifungal drug used in the treat of fungal infections, and it is white-to-pale yellow crystalline powder which is soluble in methanol and acetonitrile [3-5].

Drug profile of miconazole nitrate

It is a 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]imidazole nitric acid (Fig. 2), and it has broad-spectrum antifungal activity against various *Candida* species including *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida krusei*. It is white to off-white powder which is soluble in methanol and acetonitrile and insoluble in water [7,8].

Drug profile of tinidazole

It is 1-[2-(ethanesulfonyl)ethyl]-2-methyl-5-nitroimidazole (Fig. 3), its molecular formula is C₈H₁₃N₃O₄S and this is an antiprotozoal, antibacterial agent. Tinidazole inhibits protozoal organisms like

Trichomonas vaginalis, *Tritrichomonas foetus*, and *Entamoeba histolytica*. It is white or pale yellow crystalline powder, and it is soluble in methanol and acetonitrile [10-12].

MATERIALS AND METHODS

Optimized chromatographic conditions

Suitable HPLC equipped with ultraviolet (UV)-visible detector was used. HPLC (Waters, 2695 separation module) Software (Empower, version 2.0).

- Column: waters C18, 50 mm×4.6
- Wavelength: 210 nm
- Injection volume: 20 μl
- Column temperature: Ambient
- Flow rate: 1.0 ml/min.

Retention time of clotrimazole, miconazole nitrate, and tinidazole are 4.7, 2.9, and 3.5 min, respectively.

Chemicals and reagents

Clotrimazole, miconazole nitrate, and tinidazole were received as gift sample from ISF Analytical Laboratories, Moga, Punjab, India. The marketed formulation for the combination named as Defung Vaginal Tablets (Indica Laboratories Pvt. Ltd.) containing 20 mg of clotrimazole, 100 mg of miconazole nitrate, and 500 mg of tinidazole was purchased from local market. The solvents which were used during the method development and validation were HPLC grade methanol (S.D Fine Chemicals Ltd, Mumbai, India) and HPLC grade acetonitrile and water for HPLC (Finar Chemicals Ltd., Mumbai, India). The analytical reagent grade orthophosphoric acid (OPA) (Ranbaxy Laboratories Ltd) was used to adjust the pH of mobile phase which is filtered through a nylon 0.45 μm membrane filter paper (Gelman laboratories Mumbai, India).

RP-HPLC method

HPLC system of waters (Milford, USA) composed of 515 HPLC pump as a solvent delivery system equipped with Rheodyne injection valve with a 50 μ l loop. The chromatographic separation was performed with a Waters, 2695 separation module HPLC instrument equipped with UV-visible detector and Empower software, version 2.0. The Waters \times Bridge Stainless steel C₁₈ column (250 mm \times 4.6 mm, 5 μ m) packed with octadecylsilane chemically bounded porous silica particles was used as stationary phase for analysis. BL-220H analytical balance (Shimadzu Corporation, Japan), an ultrasonic cleaner (Frontline FS 4, Mumbai, India), and Digital pH meter (LI 612 pH analyzer, Elico Ltd., Ahmedabad) were used in the study.

Chromatographic conditions

The optimal composition of the mobile phase was determined to be acetonitrile:methanol:water, pH adjusted to 2.5 with OPA (40:30:30 v/v). The mobile phase was filtered through nylon 0.22 μ m membrane filters and was degassed to remove the air before use (30 min). Stock solution was prepared by dissolving clotrimazole, miconazole nitrate, and tinidazole (25 mg of clotrimazole and miconazole nitrate and 50 mg of tinidazole) that were weighed accurately and separately transferred into 25 ml volumetric flasks. All the drugs were dissolved in 25 ml of mobile phase to prepare standard stock solutions followed by sonication. After the immediate dissolution, sonicate the solution and make up the volume up to the mark with mobile phase. These standard stock solutions were observed to contain 1 mg/ml of clotrimazole and miconazole nitrate and 2 mg/ml of tinidazole. Appropriate volume from this solution was further diluted to get appropriate concentration levels according to the requirement. From the above stock solutions, dilutions were made in the concentration range of 4.8, 6.4, 8, 9.6, and 11.2 μ g/ml for clotrimazole; 32, 36, 40, 44, and 48 μ g/ml for miconazole nitrate; and 160, 180, 200, 220, and 240 μ g/ml for tinidazole. A volume of 50 μ l of each sample was injected into column.

Preparation of mobile phase

Mobile phase was prepared by mixing acetonitrile (HPLC grade), methanol (HPLC grade), and water (HPLC grade) pH 2.5 (55:25:20 v/v). Mixture was shaken vigorously and sonicated for 30 min before use. The pH was adjusted by OPA using pH meter (Eutech Instruments, Singapore). The prepared mobile phase was passed through 0.22 μ m membrane filter (Millipore, USA).

Preparation of stock solutions

Stock solutions and sample solutions of clotrimazole, miconazole nitrate, and tinidazole and its ternary mixture were prepared by adding accurately weighed 25 mg of clotrimazole and miconazole nitrate and 50 mg of tinidazole separately in 25 ml volumetric flask containing 15 ml of mobile phase. The flasks were then sonicated for 10 min, and the volume was made up to the mark using mobile phase. These standard stock solutions were containing 1000 μ g/ml of clotrimazole and miconazole nitrate and 2000 μ g/ml of tinidazole. Stock solutions were used to prepare desired concentration range as per sample calibration range.

Preparation of sample solutions

Twenty tablets were weighed and emptied. The equivalent weight was calculated, and according to average weight, required drug was taken in volumetric flask to obtain 20 mg of clotrimazole, 100 mg of miconazole nitrate, and 500 mg of tinidazole in single dilution, i.e., according to the label claim of the formulation. The dilutions were first sonicated and then filtered through 0.22 micron filter paper.

Preparation of calibration curve

The test concentrations for clotrimazole, miconazole nitrate, and tinidazole in HPLC method development were found to be 8 μ g/ml, 40 μ g/ml, and 200 μ g/ml, respectively. The calibration curve was prepared by injecting the concentrations of 6.4–9.6 μ g/ml of clotrimazole, 32–48 μ g/ml of miconazole nitrate, and 160–240 μ g/ml of tinidazole in ternary mixture solution manually in triplicate to the HPLC

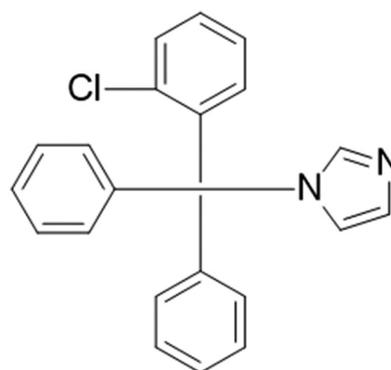


Fig. 1: Structure of clotrimazole [2]

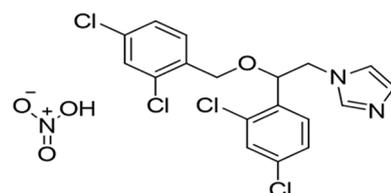


Fig. 2: Structure of miconazole nitrate [6]

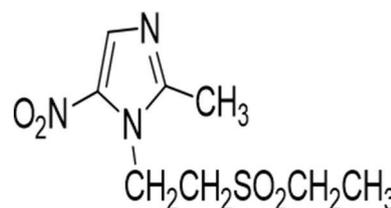


Fig. 3: Structure of tinidazole [9]

system at detection wavelength of 210 nm. Mean of n=5 determinations was plotted as the standard curve. The calibration curve was tested by validating it with interday and intraday measurements. Linearity, accuracy, and precision were determined for both interday and intraday measurement.

Method validation**Linearity**

The methods were validated according to the International Conference on Harmonization Q2B guidelines (2005) for validation of analytical procedures to determine the linearity, sensitivity, precision, and accuracy for each analyte. Calibration curves were generated with appropriate volumes of working standard solutions for HPLC.

For HPLC assay method validation, the test concentrations were found to be 8 μ g/ml of clotrimazole, 40 μ g/ml of miconazole nitrate, and 200 μ g/ml of tinidazole. Linearity of the proposed method was carried out by the preparation of 80%, 90%, 100%, 110%, and 120% of clotrimazole, miconazole nitrate, and tinidazole and test concentration, i.e., 6.4, 7.2, 8, 8.8, and 9.6 μ g/ml for clotrimazole; 32, 36, 40, 44, and 48 μ g/ml for miconazole nitrate; and 160, 180, 200, 220, and 240 μ g/ml for tinidazole. The linearity was evaluated by the least square regression method using data as shown in Table 1.

Precision and accuracy

Both precision and accuracy were determined with standard quality control samples (in addition to calibration standards) prepared in triplicates at different concentration levels covering the entire linearity range. Precision is the degree of repeatability of an analytical method

under normal operational conditions. The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday) and reported as percent relative standard deviation (% RSD) for a statistically significant number of replicate measurements. The intermediate precision was studied by comparing the assays on three different days, and the results documented as standard deviation and % RSD [13].

Accuracy is the percent of analyte recovered by assay from a known added amount. For the measurement of accuracy, data from nine determinations over three concentration levels covering the specified range were determined. The samples containing 80%, 100%, and 120% of test concentrations were spiked with 100% of standard solution in HPLC, and percentage recovery was calculated [14,15] as shown in Table 2.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is defined as the lowest concentration of an analyte that an analytical process can reliably differentiate from background levels. The LOQ is defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision, and variability [15].

The LOD and LOQ were calculated as,

$$\text{LOD}=3.3\sigma/S$$

$$\text{LOQ}=10\sigma/S$$

Where σ is the standard deviation of the lowest standard concentration and S is the slope of the standard curve as shown in Table 1.

Stability

The stability of clotrimazole, miconazole nitrate, and tinidazole in mobile phase was assessed by analyzing the sample concentrations at 1 h, 4 h, 9 h, 24 h, 48 h, and 60 h. The concentrations used in this study were 8 $\mu\text{g/ml}$ for clotrimazole, 40 $\mu\text{g/ml}$ for miconazole nitrate, and 200 $\mu\text{g/ml}$ for tinidazole prepared from stock solution of pure form and powdered capsule sample, respectively. Six replicate samples for each concentration were assayed at each time point. The stability was tested over a period of 3 days at room temperature.

Robustness

The effect of intentional variation in analytical conditions such as detection wavelength, mobile phase flow rate, and mobile phase composition on the retention time, and peak area was investigated one by one in Table 1.

Analysis of marketed tablets formulation by HPLC

Accurately weighed powder sample equivalent to 20 mg of clotrimazole, 100 mg of miconazole nitrate, and 500 mg of tinidazole was dissolved in a 50 ml volumetric flask containing mobile phase. The solution was kept for sonication for 20 min, filtered through Whatman Filter Paper No. 41. Aliquot of this solution was diluted to produce the concentration of 8 $\mu\text{g/ml}$ for clotrimazole, 40 $\mu\text{g/ml}$ for miconazole nitrate, and 200 $\mu\text{g/ml}$ for tinidazole (n=6).

RESULTS

RP-HPLC spectrophotometric methods were developed for clotrimazole, miconazole nitrate, and tinidazole which can be conveniently employed for routine analysis in pharmaceutical dosage forms and will eliminate unnecessary tedious sample preparations. The chromatographic conditions were optimized to provide a good performance of the

Table 1: Summary of the HPLC method validation

S. No	Validation parameter	Clotrimazole	Miconazole nitrate	Tinidazole
1.	Absorption maxima, λ_{max} (nm)	210	210	210
2.	Linearity range ($\mu\text{g/ml}$)	6.4–9.6	32–48	160–240
3.	Coefficient of determination (R^2)	0.998	0.997	0.999
4.	Regression equation (y)	$Y=52494x+26711$	$Y=44103x-79083$	$Y=14390x-27218$
5.	Slope (b)	52494	44103	14390
6.	Intercept (a)	26711	79083	27218
7.	Limit of detection ($\mu\text{g/ml}$)	0.159	2.726	4.219
8.	Limit of quantification ($\mu\text{g/ml}$)	0.480	8.232	12.74
9.	Precision (% RSD)	Intraday=0.524 Interday=0.540	Intraday=0.410 Interday=0.693	Intraday=1.26 Interday=1.28
10.	Solution stability >12 h	Stable up to 24 h % RSD=0.645	Stable up to 24 h % RSD=0.65	Stable up to 24 h % RSD=0.756
11.	Robustness (% RSD)	complies	complies	Complies
	Flow plus	0.460	0.640	0.733
	Flow minus	0.315	0.454	0.809
	Organic plus	0.665	0.598	0.527
	Organic minus	0.669	0.775	0.336
	Wavelength plus	0.201	0.448	0.393
	Wavelength minus	0.315	0.566	0.482

RSD: Relative standard deviation, HPLC: High-performance liquid chromatography

Table 2: Recovery study by HPLC methods

Method	Drug	Amount present ($\mu\text{g/ml}$)	Amount spiked ($\mu\text{g/ml}$)	Percent recovery*
HPLC method	Clotrimazole	6.4	8	100.31
		8	8	96.59
		9.6	8	102.51
	Miconazole nitrate	32	40	102.82
		40	40	99.98
		48	40	99.93
	Tinidazole	160	200	100.31
		200	200	99.79
		240	200	99.72

*Average of six determinations, HPLC: High-performance liquid chromatography

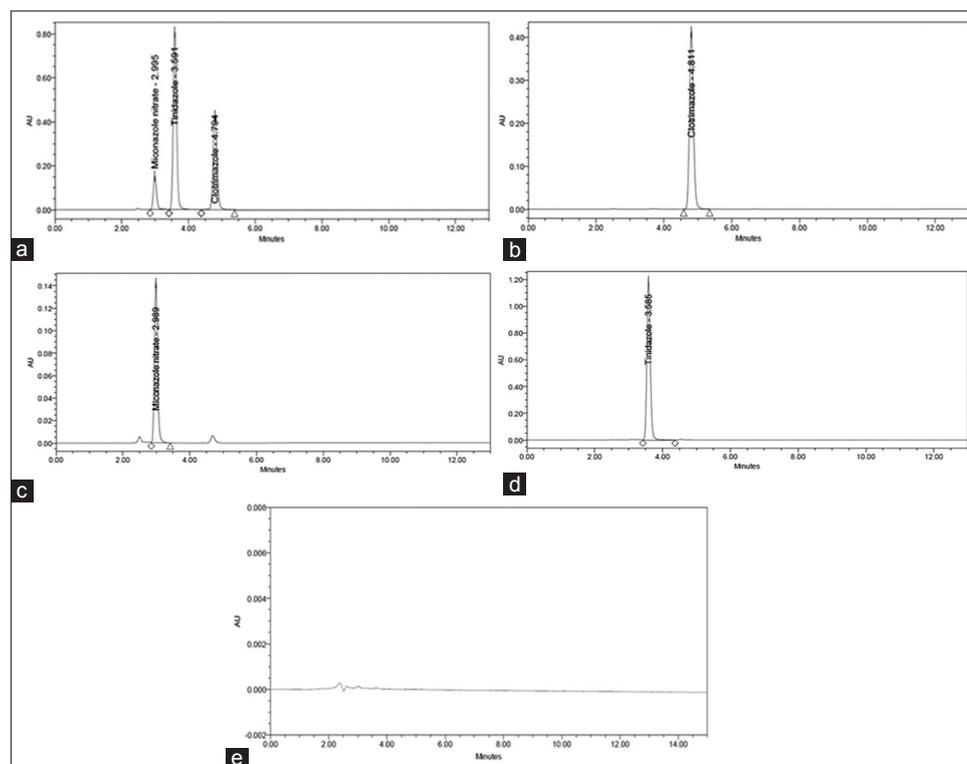


Fig. 4: (a) Chromatograms of clotrimazole, miconazole nitrate, and tinidazole. (b) Chromatogram of clotrimazole at test concentration. (c) Chromatogram of miconazole nitrate at test concentration. (d) Chromatogram of tinidazole at test concentration, (e) Chromatogram of blank

Table 3: System suitability studies of clotrimazole, miconazole nitrate, and tinidazole in optimized RP-HPLC compared with USP limits

S.No	Parameters	USP limit	Clotrimazole	Miconazole nitrate	Tinidazole
1.	Conc. (%)	-	100%	100%	100%
2.	Retention time	-	4.834	3.003	3.558
3.	Average peak area of 6 injection	-	3517422	1051242	5759661
4.	% RSD of peak area of 6 injections	≤2.0	0.425	0.569	0.676
5.	Peak asymmetry (at 10% peak height)	≤1.5	1.69	1.73	1.52
6.	Mean number of theoretical plate	≥2000	2218	2289	2434
7.	Final retention time	-	4.794	2.995	3.591

RSD: Relative standard deviation, RP-HPLC: Reversed-phase high-performance liquid chromatography

assay. The retention time of clotrimazole, miconazole nitrate, and tinidazole in tablets formulation was found to be 4.7 min, 2.9 min, and 3.5 min, respectively. The chromatograms have been shown in Fig. 4a. A five-point calibration curve was constructed with working standards and was found linear for each of the analyte over their calibration ranges. The slopes were calculated using the plot of drug concentration versus area of the chromatogram. The developed HPLC method was accurate, precise, reproducible, and very sensitive as shown in below Fig 4.

All the method validation parameters are well within the limits as specified in the ICH Q2B guidelines as shown in Tables 1 and 2 lists, the percent recovery (content uniformity) of all the drugs in the commercial formulations by HPLC methods. Moreover, the % RSD (less variation) shows good precision of developed method [16]. The calculated LOQ and LOD concentrations confirmed that the methods were sufficiently sensitive. Various system suitability parameters are shown in Table 3. The methods were suitably employed for assaying all the drugs in commercial marketed formulation (Table 4).

DISCUSSION

All the method validation parameters were well within the limits as specified in the ICH Q2B guidelines. Moreover, the % RSD (less variation) showed good precision of developed method. The calculated LOQ and

Table 4: Assay of marketed formulation Defung vaginal tablets by HPLC methods

Drug	Label claim (mg)	Amount found (mg)	% assay
Clotrimazole	20	21.443	99.83
Miconazole nitrate	100	100.501	100.05
Tinidazole	500	500.355	100.12

HPLC: High-performance liquid chromatography

LOD concentrations confirmed that the methods were sufficiently sensitive. Various system suitability parameters are shown which show that the method is simple, accurate rapid, and precise. The method was suitably employed for assaying all the drugs in commercial marketed formulation. Hence, the developed can be used for further studies, and it helps researches to determine the drug content in formulation.

CONCLUSION

Simple, rapid, accurate, and precise RP-HPLC methods have been developed and validated for the routine analysis of clotrimazole, miconazole nitrate, and tinidazole in active pharmaceutical ingredient and tablets dosage forms. The method is suitable for the simultaneous

determination of clotrimazole, miconazole nitrate, and tinidazole in multicomponent formulations without the interference of each other. The developed method is suggested for routine and quality control analysis of the investigated drugs in ternary component pharmaceutical preparations. The amount found from the proposed methods is in good conformity with the label claim of the formulation. Furthermore, the value of standard deviation and coefficient of variation calculated are acceptably low, indicating the suitability of the proposed methods for the routine estimation of tablets dosage forms.

AUTHORS' CONTRIBUTION

Deepika Sharma: Carried out the research work. Komal Gupta: Assisted in drafting the manuscript. Dr. Pooja Chawla: Research Supervisor.

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CONFLICTS OF INTERESTS

The authors confirm that this article content has no conflict of interest.

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