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Original Article

A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF AZITHROMYCIN, FLUCONAZOLE AND ORNIDAZOLE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Objective: The objective of the study was to develop and validate a new rapid and more sensitive Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the simultaneous estimation of azithromycin, fluconazole and ornidazole in bulk and pharmaceutical dosage forms.

Methods: Separation was achieved with a cap cell pack C18 column (4.6 x 250 mm, 5µ) with an isocratic mobile phase containing a mixture of acetonitrile and phosphate buffer pH 4.8 [adjusted with *ortho*-phosphoric acid] (50:50 % v/v) at the flow rate of 1 ml/min and detection was monitored at 210 nm.

Results: The retention time (Rt) of azithromycin, fluconazole and ornidazole were found to be 4.82±0.01, 5.25±0.01 and 6.33±0.01 min respectively. The precision was found with<1.5% of %RSD. The calibration curve was linear over the concentration ranging from 500-1000 µg/ml for azithromycin, 75-150 µg/ml for fluconazole and 375-750 µg/ml for ornidazole with the correlation coefficient (r^2) of 0.999. The percentage recovery was found to be within the specified range i.e., 98-102 % for three drugs. Limit of detection (LOD) was found to be 5.810, 1.790 and 4.924 µg/ml, whereas Limit of quantification limits (LOQ) was found to be 9.834, 2.667 and 7.980 µg/ml, respectively.

Conclusion: A simple isocratic liquid chromatographic method was developed and validated for simultaneous estimation of azithromycin, fluconazole and ornidazole in their formulations. Due to its simplicity, rapidness and specificity, it can be applied for routine quality control analysis of these drugs.

Keywords: Azithromycin, Fluconazole, Ornidazole, Method development, RP-HPLC

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INTRODUCTION

Azithromycin is an antibiotic useful for the treatment of a number of bacterial infections, including middle ear infections, strep throat, pneumonia, traveler's diarrhea and certain other infections. It is also used for sexually transmitted infections such as chlamydia and gonorrhea. It is chemically derived from erythromycin; it acts on bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. Thereby, inhibits peptidyl transferase activity and interferes with amino acid translocation during the process of translation.

Fluconazole is an anti-fungal medication which is being used in the number of fungal infections. It is primarily used in the treatment of candidiasis, blastomycosis, coccidiomycosis, cryptococcosis, histoplasmosis, dermatophytosis and pityriasis versicolor. It is a drug of choice in prevention of candidiasis during organ transplantation.

Ornidazole is a drug that cures some protozoan infections. It has been investigated for use in Crohn's disease after bowel resection. It is used for the treatment of stomach, intestinal, urinary tract and genital infections. Formation of redox intermediate (an intracellular metabolite) is believed to be the key component responsible for killing microorganisms.

Combination of azithromycin, fluconazole and ornidazole were co-administered to treat a variety of skin infections such as eczema, fungal skin infections including ringworm etc. Combination therapy commonly administered in the trade name of AF-kit (azithromycin-1000 mg; ornidazole-750 mg and fluconazole-150 mg) (fig. 1). As these drugs are frequently co-administered in various infections, an effective, simple and specific analytical method is required for simultaneous estimation, thereby it will be helpful in the quantification of drugs in the formulation as well as in the biological matrices. An extensive literature survey suggests that development of various analytical techniques such as ultraviolet (UV)-visible spectroscopy [1-6], HPLC [6-24], HPTLC [25-28] and LCMS [29-32] for quantification of these drugs either in individual or combination with other drugs. Recently, Krishna *et al.,* [21] reported an RP-HPLC method with the help of sodium dihydrogen *ortho*-phosphate (pH-5.2): acetonitrile in the ratio of 70:30 (%v/v) as mobile phase. Another study by Arunya *et al.*, [24] reported a stability-indicating RP-HPLC method for simultaneous estimation of these drugs with the help of procaine hydrochloride as an internal standard (IS). In the present study, we attempted to achieve a more precise and simple HPLC method by varying the chromatographic conditions. In our study, the separation and quantification were achieved with the help of phosphate buffer (pH-4.8): acetonitrile in 50:50 % v/v ratio as a mobile phase without using any IS. Moreover, the rapid elution with shorter runtime was observed at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min for azithromycin, fluconazole and ornidazole, respectively. In addition, the proposed method was validated as per ICH guidelines in terms of various parameters, and the results were found to be within acceptance criteria. In these studies, the method was found to be simple, rapid and more precise.

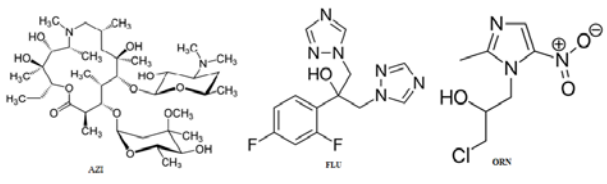

Fig. 1: Chemical structures of azithromycin, fluconazole and ornidazole

MATERIALS AND METHODS

Instrumentation

Lab India UV-3000+double beam UV-Visible spectrophotometer was used to carry out absorption studies and the data were recorded by UV-Win software. Standard cuvettes of 10 mm path length were used for analysis. Cyber lab LC-100 HPLC system is accomplished with UV-detector, quantitative HPLC was performed on an isocratic mode using cap cell pack C18 (4.6 x 250 mm, 5µ) column with 20 µL injections of the sample loop. The output signal was monitored and integrated using Cyber lab LC-100 software. Lifecare ultra-sonic cleaner was used to sonicate the standard and sample solutions.

Chemicals and reagents

Standard drugs of azithromycin, fluconazole and ornidazole were obtained as gift samples from Cipla Laboratory, Malapur and Aurobindo Pharmaceuticals, Hyderabad. Market formulation (AF-kit, Madras Pharmaceuticals, Chennai) was procured from the local market. HPLC grade acetonitrile and methanol were obtained from Merck Life Sciences, Mumbai, India. Analytical grade solvents and other chemicals were acquired from SD Fine Chemicals, Mumbai, India. Water was obtained from millipore with milli Q system, filtered through 0.45 µ nylon membrane for the HPLC experiments.

Preparation of phosphate buffer

Phosphate buffer was prepared by dissolving 13.6 g of potassium dihydrogen *ortho*-phosphate in 1000 ml of water (HPLC grade) and pH was adjusted to 4.8 with *ortho*-phosphoric acid and solution was filtered through 0.45 µ millipore nylon filter.

Preparation of the mobile phase

Acetonitrile: phosphate buffer (pH-4.8) in the ratio of 50:50 % v/v was prepared and it was filtered through 0.45 µ millipore nylon filter. The solution was degassed with ultrasonic cleaner for 15 min. The resultant solution was used as the mobile phase.

Chromatographic conditions

The method was developed by using a cap cell pack C18 column (4.6 x 250 mm, 5µ) with an isocratic mobile phase which consists a mixture of acetonitrile and potassium dihydrogen *ortho*-phosphate buffer (pH-4.8 adjusted with *ortho*-phosphoric acid) in 50:50 % v/v ratio. The mobile phase was filtered through 0.45 µ millipore nylon filter under vacuum filtration. Flow rate of the mobile phase was adjusted to 1 ml/min. The eluted compounds were detected at the wavelength of 210 nm. The sample injection volume was 20 µl.

Preparation of a standard mixture of azithromycin, fluconazole and ornidazole

Transfer 100 mg of azithromycin, 15 mg of fluconazole and 75 mg of ornidazole into a 100 ml of volumetric flask and add 70 ml of diluent. Then it was sonicated for 15 min and the volume made up to 100 ml with diluent. The resulted solution (20 µl) was injected into the HPLC system by employing optimized chromatographic conditions.

Preparation of sample mixture of azithromycin, fluconazole and ornidazole

Twenty tablets of each of azithromycin, fluconazole and ornidazole (AF-Kit) were weighed and the average weight of each tablet was determined individually. The tablets were crushed into a fine powder, accurately weighed tablet powder equivalent to 100 mg (0.1121 g) of azithromycin, 15 mg (0.239 g) of fluconazole and 75 mg (0.169 g) of ornidazole and transferred into a clean 100 ml volumetric flask. Add 70 ml of diluents, and then sonicated for 15 min to dissolve, made up to the volume with diluents. The resulted solution was filtered through 0.45µ membrane filter and then above resulted solution 20 µl was injected into the HPLC system.

Selection of detection wavelength

The standard stock solution of azithromycin, fluconazole and ornidazole in the concentration of 10 µg/ml was prepared and each solution was scanned in UV range (200-400 nm) in 10 mm path length against the solvent blank. The overlain spectrum of three drugs was recorded against solvent blank.

Method validation

Method validation was performed using standard and sample solutions of analytes as per ICH guidelines for proposed method [33-35]. The following validation parameters performed such as specificity, linearity, precision, accuracy, robustness, LOD and LOQ etc.

RESULTS

The purpose of the present study was to develop a rapid and sensitive RP-HPLC method for the simultaneous estimation of azithromycin, fluconazole and ornidazole in their dosage form using cap cell pack C18 analytical column with UV detection.

Selection of detection wavelength

The study of the absorption spectrum for three drugs revealed that well-defined λ_{max}. The iso-absorptive point was obtained at 210 nm for three drugs at the given concentration. Obtained wavelength maxima and iso-absorptive point for the three drugs were used for the simultaneous estimation by using RP-HPLC method. The overlain spectrum of azithromycin, fluconazole and ornidazole was shown in fig. 2.

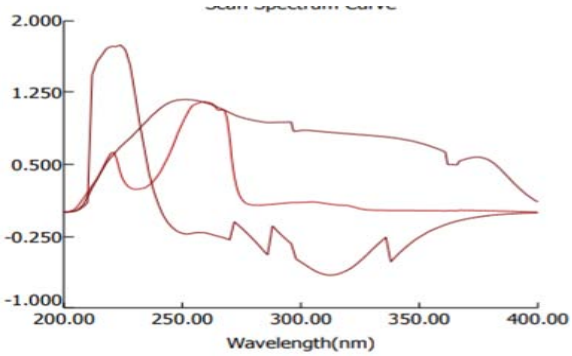

Fig. 2: Overlain spectrum of azithromycin, fluconazole and ornidazole

Method optimization

Optimization of chromatographic conditions for isocratic RP-HPLC detection, parameters such as mobile phase composition, pH (4.8-6.3) and flow rate (1 ml/min±0.2) were varied for system suitability studies. The variation in the mobile phase led to considerable changes in the chromatographic parameters like asymmetric factor, capacity factor and retention time (Rt). Varying in pH showed that optimized conditions were reached at pH 4.8, producing well resolved and sharp peaks for three drugs. Henceforth, in the present method, the acetonitrile and phosphate buffer (50:50 % v/v) (pH-4.8) was used as a mobile phase with 1.0 ml/min flow rate as optimal conditions. The detection of peaks achieved at 210 nm for the three analyte drugs. For quantitative determination of azithromycin, fluconazole and ornidazole in formulations, initially mixed standard solutions in appropriate concentrations were injected into the column and Rt's of azithromycin, fluconazole and ornidazole was found to be 4.82±0.01, 5.25±0.01 and 6.33±0.01 min, respectively (fig. 3).

Method validation

Method validation was performed as per ICH guidelines for simultaneous determination of the azithromycin, fluconazole and ornidazole. The results of parameters such as specificity, linearity, precision, accuracy, robustness, detection limit, quantification limit were described in the following sections.

Specificity

No interference of additives peak was found in the chromatogram for three drugs in tablet formulation, which indicates the proposed method is specific. Blank determination also performed where no appearance or interference of peaks observed.

Linearity

Standard solutions at six different concentration levels ranging from 500-1000 µg/ml for azithromycin, 75-150 µg/ml for fluconazole and 375-750 µg/ml for ornidazole were prepared and analyzed in order to demonstrate the linearity relationships. For linearity study, the standard solutions were prepared as per label claim amount in the tablets. The regression curve was obtained by plotting the peak area v/s concentration of each analyte (fig. 4-6). It was obtained by least-squares method [15]. The correlation coefficient (r^2) values were found to 0.999 for all the drugs. Detailed Linearity data were summarized in table 1.

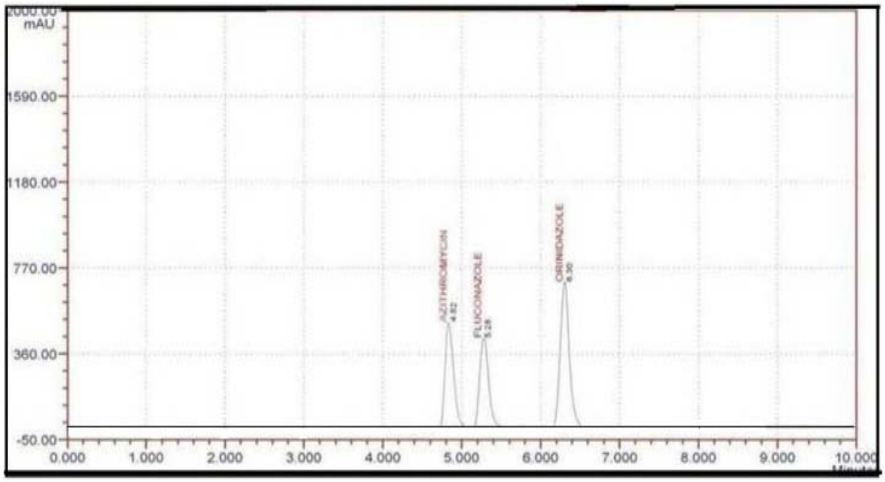

Fig. 3: Standard chromatogram of azithromycin, fluconazole and ornidazole in acetonitrile and phosphate buffer pH-4.8 (50:50%v/v)

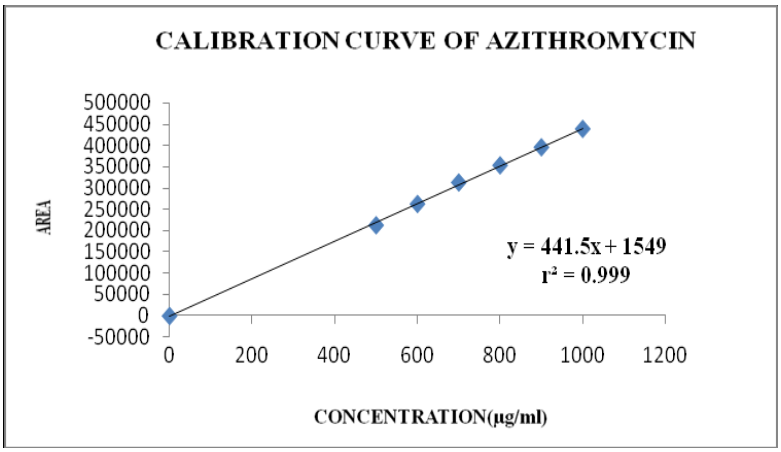

Fig. 4: Calibration curve of azithromycin (1000-500 µg/ml)

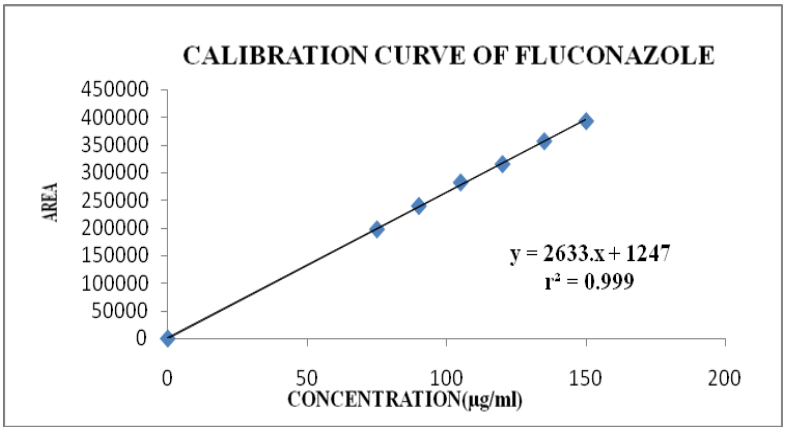

Fig. 5: Calibration curve of fluconazole (75-150 µg/ml)

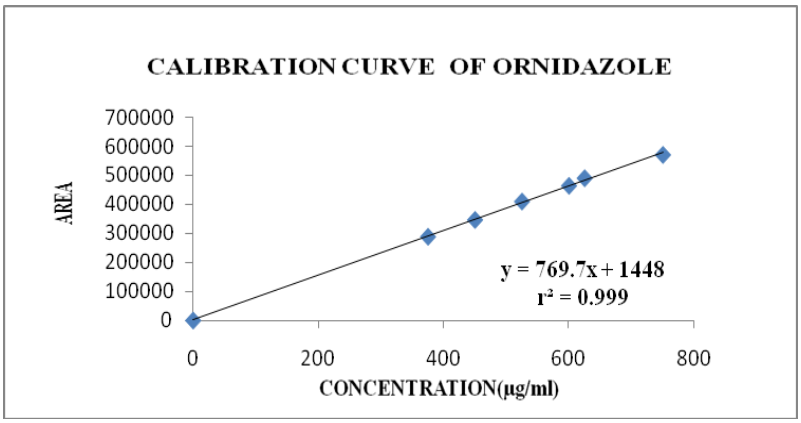

Fig. 6: Calibration curve of ornidazole (375-750 µg/ml)

Table 1: Linearity data of azithromycin, fluconazole, ornidazole

Analyte	Concentration (µg/ml)	Peak area (mV)	Linear regression equation
	500	212776	
Azithromycin	600	262627	y = 439.5x+1549
	700	312991.6	r^2 = 0.999
	800	353358.8	
	900	395606.7	
	1000	438629	
	75	197526.9	
Fluconazole	90	239958.4	y = 2643x+1247
	105	282508.2	r^2 = 0.999
	120	315641.9	
	135	357006.2	
	150	438629	
	375	288696	
Ornidazole	450	346460.7	y= 772.2x+1448
	525	409934.6	r^2 = 0.999
	600	464002.2	
	625	490068.5	
	750	570405.9	

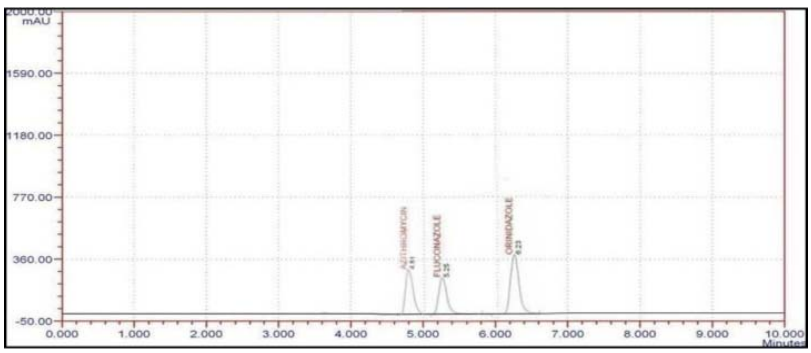

Fig. 7: Chromatogram of 50% standard addition (level-1)

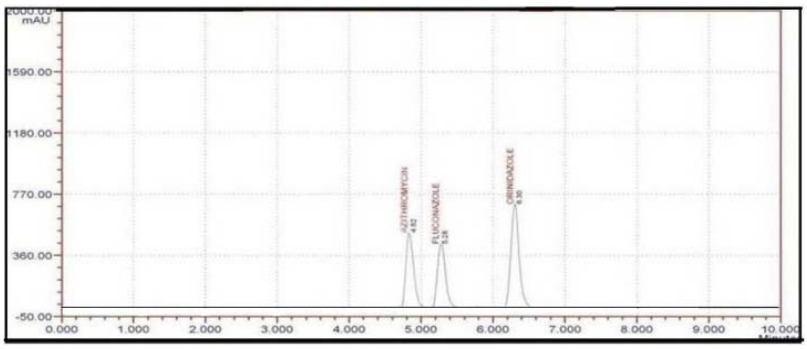

Fig. 8: Chromatogram of 100% standard addition (level-2)

Accuracy

Standard addition experiments were employed for accuracy studies in which the percent (%) recovery was determined. Accuracy of the method was evaluated in triplicate at three concentration levels, i.e. 50 %, 100 % and 150 % of target concentration and the percentages of recoveries were calculated. Resulted chromatograms at these levels were shown in fig. 7-9. Percentage recoveries of three drugs found in between 98.3-101.2 %, which indicates the results obtained were within the potency range (table 2-4).

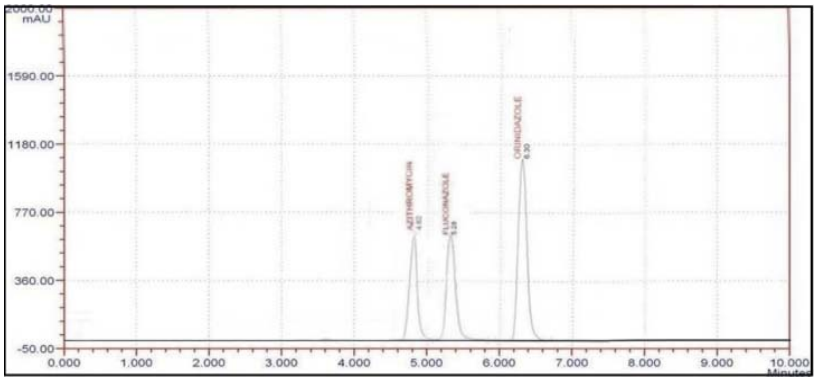

Fig. 9: Chromatogram of 150% standard addition (level-3)

Table 2: Accuracy data of azithromycin (n = 3)

S. No.	Spiked level	Sample area	Sample height	% Recovery	% mean recovery±SD
	50%	228316	24846	101.4	
1	50%	227484	24656	101.4	101.2±0.288
	50%	225988	24389	100.9	
	100%	438654	49692	99.9	
2	100%	438962	49343	99.2	99.3±0.472
	100%	446756	49654	99.0	
	150%	665493	74538	98.1	
3	150%	668649	74832	98.2	98.3±0.264
	150%	667482	74983	98.6	

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 3: Accuracy data of fluconazole (n= 3)

S. No.	Spiked level	Sample area	Sample height	% Recovery	%mean recovery±SD
1	50%	205368	21689	101.0	100±0.0577
	50%	204654	21594	100.0	
	50%	201844	21863	100.0	
2	100%	395978	43189	100.3	
	100%	393878	43163	98.5	99.8±1.209
	100%	398398	43258	100.8	
3	150%	603246	64783	99.1	99±0.0577
	150%	605685	64685	99.1	
	150%	609483	64981	99	

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 4: Accuracy data of ornidazole (n = 3)

S. No.	Spiked level	Sample area	Sample height	% Recovery	%mean recovery±SD
1	50%	295162	35021	100.8	
	50%	293853	35683	99.8	100.1±0.578
	50%	293345	35942	99.8	
2	100%	576369	70042	100.9	
	100%	579209	70861	101.1	100.5±0.871
	100%	570405	70932	99.5	
3	150%	864543	105882	99.1	99.5±0.378
	150%	868347	105936	99.8	
	150%	869543	105856	99.7	

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Precision

Precision of the method was determined by injecting the standard and sample solutions of azithromycin, fluconazole and ornidazole separately for six times and measured % RSD with the help of peak area for all six injections. System precision was established by injecting six replicate injections of standard solutions into the chromatographic system by maintaining the optimized conditions. Method precision was established by injecting six replicate injections of sample solution into the chromatographic system by

maintaining the optimized conditions. In both cases, the % RSD found to be <2%, which indicate the proposed method was more precise. The precision data were summarized in table 5 and 6.

Robustness

Robustness experiments were carried by altering chromatographic conditions such as flow rate and detection wavelength to demonstrate any deliberate changes in the proposed method. The changes were made in the chromatographic conditions, *viz*. change in flow rate by ±0.2 ml/min and change in the wavelength ±5 °C. The % RSD was found <2% for three drugs even slight change of flow rate as well as detection wavelength (table 7, 8 and 9). As there were no significant variations in elution time for all the three drugs, indicate the method was robust.

Table 5: System precision data of azithromycin, fluconazole and ornidazole (n=3)

S. No.	Rt			Peak area			Height of peak		
	Azithromycin	Fluconazole	Ornidazole	Azithromycin	Fluconazole	Ornidazole	Azithromycin	Fluconazole	Ornidazole
1	4.81	5.25	6.33	438629	383393	560485	49692	43189	70042
2	4.82	5.26	6.34	438732	383871	564479	49343	43163	70861
3	4.81	5.26	6.32	429781	383878	566369	49461	43258	70932
4	4.82	5.25	6.32	429658	386958	568349	49461	43281	70865
5	4.81	5.24	6.34	429268	386215	566332	49586	43856	70832
6	4.82	5.25	6.33	429658	386115	565891	49856	43328	70156
mean±SD	4.83	5.26	6.33	432621±3268.5	385071±232.5	565317±1639.1	-	-	-
% RSD	-	-	-	0.75	0.06	0.28	-	-	-

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 6: Method precision data of azithromycin, fluconazole and ornidazole (n=3)

S. No.	Azithromycin		Fluconazole		Ornidazole	
	Rt	Peak area	Rt	Peak area	Rt	Peak area
Injection-1	4.81	438654	5.25	395978	6.33	576369
Injection-2	4.81	438962	5.26	396710	6.33	579209
Injection-3	4.82	446756	5.25	396748	6.33	576432
Injection-4	4.82	448756	5.25	397864	6.34	578659
Injection-5	4.81	448851	5.25	396324	6.33	578956
Injection-6	4.81	446231	5.26	395224	6.32	576354
mean±SD	4.82	444701.0±3031.6	5.24	396474.6±89.41	6.33	577713.1±932
% RSD	-	0.68	-	0.02	-	0.16

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 7: Robustness data of azithromycin

Factor	Level	Mean±SD of area	%RSD
Flow rate	0.8	427494.5±1579.11	0.36
(ml/min)	1	438808±217.78	0.04
	1.2	428998.5±494.26	0.11
Wavelength	205	554261±84.852	0.01
(nm)	210	435941.7±3800.6	0.87
	215	544333±13231.3	0.16

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 8: Robustness data of fluconazole

Factor	Level	Mean±SD of area	%RSD
Flow rate	0.8	385962.5±744.58	0.19
(ml/min)	1	396168±268.70	0.06
	1.2	386261.5±451.84	0.11
Wavelength(nm)	205	484532.5±1274.91	0.26
	210	383603±56.56	0.01
	215	485995±500.63	0.10

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Table 9: Robustness data of ornidazole

Factor	Level	Mean±SD of area	%RSD
Flow rate	0.8	567256±2265.5	0.39
(ml/min)	1	577789±2007.82	0.34
	1.2	568794.5±207.18	0.03
Wavelength	205	625212.5±566.39	0.09
(nm)	210	563655±268.7	0.04
	215	625656.5±371.23	0.05

n = number of determinations, SD = Standard deviation, % RSD = % Relative standard deviation

Limits of detection and quantification (LOD and LOQ)

The sensitivity of the method was measured by calculating the LOD and LOQ. LOD and LOQ were assessed as per ICH guidelines, i.e., at signals to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations (fig. 10 and 11). The linear regression equation was plotted for concentration v/s area of the peak. The standard deviation of y-intercepts and slope were considered for determination of sensitivity ranges for LOD and LOQ (table 10 and 11).

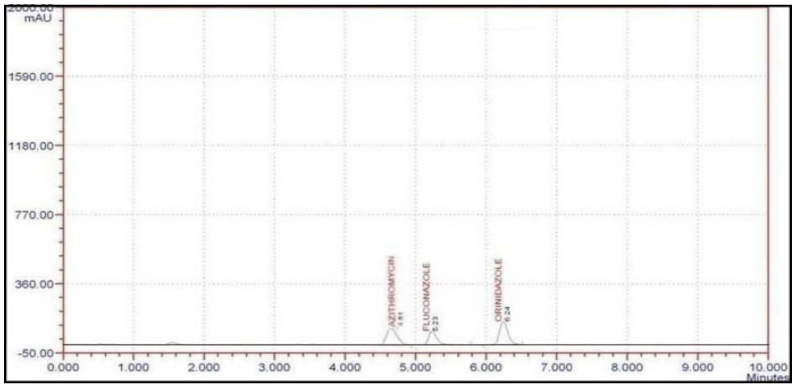

Fig. 10: Chromatogram of LOD studies

Table 10: LOD data of azithromycin, fluconazole and ornidazole (n = 3)

S. No.	Drug	Peak area	LOD (µg/ml)
1	Azithromycin	2157	5.810
2	Fluconazole	9950	1.790
3	Ornidazole	57739.2	4.924

n = number of determinations.

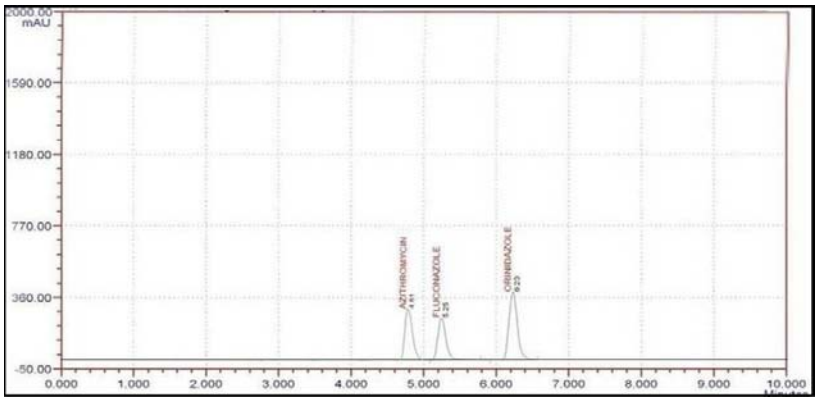

Fig. 11: Chromatogram of LOQ studies

Table 11: LOQ Data of azithromycin, fluconazole and ornidazole (n = 3)

S. No.	Drug	Peak area	LOQ (µg/ml)
1	Azithromycin	3878	9.834
2	Fluconazole	14887	2.667
3	Ornidazole	96232.1	7.980

n = number of determinations.

DISCUSSION

The present study describes the development and validation of a RP-HPLC method for simultaneous estimation of azithromycin, fluconazole and ornidazole in bulk drug and in their tablet dosage form. The separation was achieved on cell pack C18 column (4.6 × 250 mm, 5µ) with UV detection. The method involves with acetonitrile and phosphate buffer pH-4.8 (50:50 % v/v) as mobile phase. The flow rate was monitored at 1.0 ml/min with an injection volume 20 µl. The separation was achieved by UV-detection wavelength at 210 nm. The peaks were eluted at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min for azithromycin, fluconazole and ornidazole, respectively which was found with a short time of elution and well resolved to that of the reported method [22].

The decrease in buffer content (aqueous content) as well as adjusting pH at 4.8 of the mobile phase system resulted in rapid elution of fluconazole (RT-5.25 min). The system suitability parameters such as theoretical plates and tailing factor were found to be 8310, 1.0 (azithromycin), 8288, 1.8 (fluconazole) and 11846,

1.5 (ornidazole). Linearity was found over the concentration range of 500-1000 µg/ml for azithromycin, 75-150 µg/ml for fluconazole and 375-750 µg/ml for ornidazole with a correlation coefficient (r^2) of 0.999 for three drugs. The method found to be more accurate with % recoveries of 100-102 %, 99-100 % and 98-102 % for azithromycin, fluconazole and ornidazole, respectively. LOD values were 5.810 µg/ml (azithromycin), 1.790 µg/ml (fluconazole) and 4.924 µg/ml (ornidazole). LOQ values were 9.834 µg/ml (azithromycin), 2.667 µg/ml (fluconazole) and 7.980 µg/ml (ornidazole). The present liquid chromatographic method was found that it is as more appropriate for simultaneous estimation of such multi-component (three-drug combinations) pharmaceutical dosage forms.

CONCLUSION

A simple, accurate and precise RP-HPLC method has been developed for the estimation of azithromycin, fluconazole and ornidazole in tablet dosage form using UV-detector. A RP-cell pack C18 column (4.6 × 250 mm, 5 µ) with a mobile phase consisting of acetonitrile and phosphate buffer (pH-4.8) (50:50 % v/v) at 1 ml/min flow rate was used and the effluents were monitored at 210 nm. The results obtained by the proposed method were found as highly resolved, rapid with shorter runtime over previous reported methods. The peaks were eluted at 4.82±0.01, 5.25±0.01 and 6.33±0.01 min, respectively. The percentage of recoveries were found as 100-102 %, 99-100 % and 98-102% with accepted limits of % RSD (<2%) for three drugs, respectively. The present RP-HPLC method developed was well suitable for routine analysis of these drugs in their pharmaceutical formulation and it can be also applicable to biological samples.

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AUTHORS CONTRIBUTIONS

All authors contributed equally to this manuscript

CONFLICTS OF INTERESTS

The authors declare no conflict of interest. It has not meant to publish elsewhere. And it has not meant simultaneously presented for publication elsewhere. All authors have decided to the submission to the journal.

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