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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF MICONAZOLE AND ORNIDAZOLE IN THEIR COMBINED MARKETED DOSAGE FORM

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ABSTRACT
A simple, precise, accurate, sensitive, specific and reliable stability indicating RP-HPLC method was developed for the simultaneous estimation of Miconazole (MIC) and Ornidazole (ORN) in pharmaceutical dosage form. The method was developed with mobile phase containing buffer (0.05M potassium dihydrogen ortho phosphate, ph-3.5): Methanol in the ratio of 25:75, C18 (250 x 4.6mm, 5µm) as a stationary phase and flow rate was 1 ml/min. Detection was carried out at 236nm in UV-2000 detector. The selected chromatographic conditions were found effectively to separate Miconazole and Ornidazole at 6.58 and 3.26 min respectively. The proposed method has been validated for precision, accuracy, robustness. Thus, the statistical analysis confirms that developed methods were successfully used for analysis of formulation and thereby can be used for routine analysis of drugs in Quality Control laboratories.

KEYWORDS: RP-HPLC, Miconazole, Ornidazole, Stability Indicating, Validation.

INTRODUCTION
Miconazole-1-(2-(2,4-Dichlorobenzyloxy)-2-(2,4-dichlorophenyl)ethyl)-1H-imidazole is topical imidazole antifungal agent and Ornidazole - 1-Chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl)propan-2-ol is nitro imidazole anti amoebic and anti infective agent. Structure of MIC and ORN is shown in Fig.1 and Fig. 2.[1-6] They are used in vaginitis. This combination is used synergistically by preventing the growth of fungi and increasing cellular permeability and inhibiting the growth of microorganism.This Combination is official in IP-2014, U.S.P-32; N.F.-30, B.P.-2010.[1-3] As per literature survey, methods like RP-HPLC, stability, UV spectrophotometric methods[9-14] have been reported for Miconazole and ornidazole individually. But there is no any method have been reported for stability indicating RP-HPLC method for simultaneous estimation of both the drugs in pharmaceutical dosage form. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of stability -indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, etc. and separation of drug from degradation products. Thus the objectives of this work is to develop a new sensitive stability indicating RP-HPLC method for simultaneous determination of miconazole and ornidazole in mixture. Also it is validated for market product containing miconazole and ornidazole in tablet dosage form.

MATERIALS AND METHODS
Standard Miconazole and Ornidazole were obtained as gift sample from Pharma Supply Agencies, Navrangpura, Ahmedabad; Thermoseparation (gradient) chromatograph with UV 2000 detector was used with Data Ace Software. Methanol and Acetonitrile - HPLC grade, Water - HPLC grade, Merck India Ltd., Mumbai, was used. A commercial tablet formulation CANDIFEM was purchased from local market.

Selection of Detection wavelength
Solution of 5µg/ml and 25µg/ml of each MIC and ORN were prepared and scanned over the range 200-400 nm and the spectra were recorded. Wavelength 236 nm (at which both the drugs showed good absorbance) was selected as detection wavelength (figure 3).

Selection of Mobile phase
Std stock solutionn of ORN: 25mg of ORN dissolved in 100ml with Methanol gives 250µg/ml
Std stock solution of MIC: 50mg of MIC dissolved in 100ml with Methanol gives 500µg/ml. Further 1 ml is dissolved in 10ml gives 50µg/ml.

Working Standard Preparation (Combine standard preparation)
Take 1ml from ORN stock and 1ml from MIC std stock soln and dilute with 10ml with Mobile phase (mobile phase which used for trials) (ORN-25mcg/ml, MIC-5mcg/ml). Inject above working std preparation for mobile phase selection Chromatogram in optimized mobile phase is shown in Fig. 4.

Preparation of standard and stock solution
Stock solution of the drugs prepared by dissolving 50 mg of MIC and 25 mg of ORN with 100 ml of methanol to give standard solution of MIC and ORN of 50µg/ml and 250µg/ml respectively.

Optimized Chromatographic Conditions

Column	**C_{18} (250 x 4.6mm, 5µm)**
Mobile Phase	buffer(0.05M potassium dihydrogen ortho phosphate, ph-3.5):Methanol in the ratio of 25:75
Flow rate	1 ml/min
Detection	236 nm
Column Temperature	30ºC
Retention Time	Miconazole and Ornidazole at 6.58 and 3.26 min respectively
Run Time	10min
Injection volume (loop)	20 µl

METHOD VALIDATION

Linearity
Calibration curve of MIC and ORN were chromatographed over the range of 2.5-7.5 µg/ml and 12.5-37.5µg/ml respectively. The calibration curve was linear and regression analysis was obtained. Linearity plots were shown in Fig. 5 and Fig. 6. Results for linearity are shown in table 1.

Accuracy (Recovery study)
Accuracy of an analysis is determined by calculating systemic error involved. Recovery of MIC & ORN were calculated by standard addition method at three different concentration levels of drug. Accuracy was determined at three different level 80 %, 100% and 120 % of the target concentration 5 µg/ml of MIC and 25 µg/ml of ORN in triplicate and calculating % recovery. Results are shown in table 2.

Precision
Repeatability was assessed by analyzing six injection of a homogeneous sample of 5µg/ml of MIC and 25µg/ml of ORN. Intra-day precision was performed using three different concentration 2.5µg/ml, 5µg/ml, 7.5µg/ml for MIC and 12.5µg/ml, 25µg/ml, 37.5µg/ml for ORN in triplicate at three different time interval in a day. Inter-day precision was performed using three different concentration 2.5µg/ml, 5µg/ml, 7.5µg/ml for MIC and 12.5µg/ml, 25µg/ml, 37.5µg/ml for ORN in triplicate for three consecutive days. (Table 3).

LOD and LOQ
LOD and LOQ of the drug were calculated from signal-to-noise ratio (i.e. 3.3 for LOD and 10 for LOQ) The results were shown in table 4.

Robustness
Inject working std preparation for different flow rate, different pH and different mobile phase composition:

Flow rate: +0.2ml/mint and -0.2ml/mint
Buffer pH: +0.2pH and -0.2pH
Solvent % in mobile phase: +2% solvent and -2% solvent in mobile phase.
The results were shown in table 5.

System suitability
It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like Plate number (N), Resolution (R), tailing factor, Capacity factor, HETP, Peak symmetry of samples were measured. The results were shown in table 6.

Specificity
Commonly used excipients in tablet preparation were spiked in a pre-weighed quantity of drugs and then area was measured and calculations carried out to determine the quantity of the drugs.

Assay of marketed formulation
For analysis of the tablet dosage form, twenty tablets were weighted individually and their average weight was determined after that they were crushed to fine powders. Take tablet powder equivalent to 25mg ORN/5mg MIC in to a 100ml volumetric flask. Add 60 ml Methanol. Shake for 15 minutes and sonicate for 10 minutes. Make up volume with Methanol. Filter this solution with Whatman filter paper no-1. (ORN-250µg/ml, MIC-50µg/ml).

Working sample preparation
Take 1ml from sample stock solution into a 10ml and make up with mobile phase. (ORN-25µg/ml, MIC-5µg/ml). The solution contains Miconazole and Ornidazole in the proportions of 1: 5.

The assay procedure was made in triplicate and % drug was calculated. Results are shown in table 7 and figure 6.

FORCED DEGRADATION

Acid degradation

Forced degradation in acidic medium was performed by pipette out 1ml stock solution each of Miconazole (MIC) and Ornidazole (ORN) in separate 25 ml volumetric flasks, add 5 ml of 1 N HCl to each flask. Flasks were heated at 50ºC for 2 hrs and allowed to cool at room temperature. Solutions were neutralized with 1 N NaOH and volume was adjusted to the mark with methanol. Aliquot of 1 ml was pipette out from above solutions in separate 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solutions were analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of drugs remain un-degraded were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

Base degradation

Forced degradation in basic medium was performed by pipette out 1ml stock solution each of Miconazole (MIC) and Ornidazole (ORN) in separate 25 ml volumetric flasks, add 5 ml of 1 N NaOH to each flask. Flasks were heated at 50ºC for 1 hr and allowed to cool at room temperature. Solutions were neutralized with 1 N HCl and volume was adjusted to the mark with methanol. Aliquot of 1 ml was pipette out from above solutions in separate 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solutions were analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of drugs remain un-degraded were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

Neutral Hydrolysis

Forced degradation in neutral medium was performed by pipette out 1ml stock solution each of Miconazole (MIC) and Ornidazole (ORN) in separate 25 ml volumetric flasks, add 10 ml of double distil water to each flask. Flasks were heated at 50ºC for 2 hrs and allowed to cool at room temperature. The volume was adjusted to the mark with methanol. Aliquot of 1 ml was pipette out from above solutions in separate 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solutions were analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of drugs remain un-degraded were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

Oxidative degradation

To perform oxidative stress degradation, pipette 1 ml stock solution each of Miconazole (MIC) and Ornidazole (ORN) in separate 25 ml volumetric flasks and add 5 ml of 6% H_2O_2. Flasks were heated at 50ºC for 2 hrs and allowed to cool at room temperature and volume was adjusted to the mark with methanol. Aliquot of 1 ml was pipette out from above solutions in separate 10 ml volumetric flasks and volume was adjusted to the mark with mobile phase to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solution were analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of drugs remain undegraded were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

Thermal degradation

To study dry heat degradation, 50 mg each of Miconazole (MIC) and 25 mg of Ornidazole (ORN) were weighed and transferred in separate 25 ml volumetric flasks. The solid drugs were exposed in oven at 50^0C for 2 hrs. The solids were allowed to cool and dissolved in few ml of methanol and transfer in 10 ml volumetric flask at last volume was made up to the mark of 100ml with the methanol. Aliquot of 1 ml from above solutions were transferred to separate 10 ml volumetric flasks and volume was adjusted to the mark with methanol to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solution was analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

Photolytic degradation

To study photo degradation, 50 mg each of Miconazole (MIC) and 25mg of Ornidazole (ORN) were weighed, transferred in separate petridish. The solid drugs were exposed to sunlight for 72 hrs. Furthermore, a stress degradation study in direct UV radiation was performed by exposing the solid drugs of MIC and ORN and their mixture to UV radiation at 254 or 365 nm for 2 h at room temperature.

The solids were allowed to cool and dissolved in few ml of methanol and transfer in 10 ml volumetric flask at last volume was made up to the mark of 100ml with the methanol. Aliquot of 1 ml from above solutions were

transferred to separate 10 ml volumetric flasks and volume was adjusted to the mark with methanol to obtain final concentration of 5µg/ml of Miconazole (MIC) and 25µg/ml Ornidazole (ORN) respectively. The final solution was analyzed under the proposed chromatographic conditions and chromatograms recorded. The amounts of undegraded drugs were computed using regression equation. Same procedure was carried out for Miconazole (MIC) and Ornidazole (ORN) in mixture as per above forced degradation condition.

RESULT AND DISCUSSION

The present work aimed development and validation of stability indicating RP-HPLC method for simultaneous estimation of MIC and ORN. Method was developed in mobile phase containing buffer(0.05M potassium dihydrogen ortho phosphate, ph-3.5):Methanol in the ratio of 25:75. Detection was carried out at 236 nm. Method was validated as per ICH guidelines. Linearity and regression data were shown in table 1 and Fig.4, 5. % recovery for MIC and ORN were within the range (98% - 102%). Results were shown in table 2. Hence it is found that the developed method is accurate. %RSD values were <2 for repeatability, intra-day and inter-day precision. Results were shown in table 3. So, the developed method was found to be precise. LOD and LOQ values were shown in table 4. LOD & LOQ confirms the method to be sensitive. Small changes were carried out in mobile phase and flow rate for robustness study, in that % RSD of area was found to be <2. Results were shown in table 5.So, the developed method was found to be robust. Various forced degradation conditions were performed in proposed method and it can efficiently separate all the degradation products from the drugs. % degradation values are 5% to 20% degradation of the drug substance, have been considered as reasonable and acceptable for validation of chromatographic assays. Results were shown in table 8. So, the developed method is stability indicating.

Table: 1. Statistical analysis data of calibration curve

Sr. no.	Miconazole (MIC)	Mean Peak Area *	Ornidazole (ORN)	Mean Peak Area *
1	2.5	2000.337	12.5	2263.755
2	3.75	3016.36	18.75	3410.475
3	5	4050.77	25	4577.802
4	6.25	4860.294	31.25	5510.737
5	7.5	6082.623	37.5	6864.27
	SD	98.5915	SD	100.2058
	Slope	800.6	Slope	180.8
	Regression Coefficient (r^2)	0.99854	Regression Coefficient (r^2)	0.99882

Table 2: Accuracy

Level	Sample amount	(Standard) Drug added (µg/ml)	Drug Recovered (µg/ml) [a]	% Drug Recovered ± SD
For Miconazole (MIC)				
80	2.5	2	2.0132	98.962 ± 1.127
100	2.5	2.5	2.5054	100.553 ± 0.651
120	2.5	3	2.9968	99.896 ± 0.603
For Ornidazole (ORN)				
80	125	10	10.0714	99.706 ± 1.06
100	12.5	12.5	12.5354	100.292 ± 0.66
120	12.5	15	14.9008	99.274 ± 0.53

Table 3: Intraday and Interday Precision study for MIC and ORN

	Intraday Precision (MIC)			
Conc. (µg/ml)	**Area**	**Average area**	**SD**	**%RSD**
2.5	1984.369	1987.712333	4.19242	0.21091
	1986.352			
	1992.416			
5	4018.476	4026.534333	8.06050	0.200184
	4026.53			
	4034.597			
7.5	6024.823	6041.05633	16.746122	0.277205
	6040.074			
	6058.272			
Interday Precision (MIC)				

2.5	1986.352	1990.363	4.022045	0.20207
	1990.341			
	1994.396			
5	4022.496	4030.555	8.0605	0.19998
	4030.553			
	4038.617			
7.5	6027.982	6040.088	12.1135	0.20055
	6040.074			
	6052.209			

n=Three determination.

Intraday and Interday Precision study for ORN

	Intraday Precision			
Conc. (µg/ml)	**Area**	**Average area**	**SD**	**%RSD**
12.5	2245.686	2248.4536	3.058902	0.136044
	2247.937			
	2251.738			
25	4541.284	4548.9693	7.081995	0.155683
	4550.392			
	4555.232			
37.5	6809.556	6817.6093	8.7391583	0.1281850
	6816.37			
	6826.902			
Interday Precision				
12.5	2247.937	2251.009	2.66284	0.11829
	2252.439			
	2252.652			
25	4545.83	4553.359	6.87766	0.15104
	4554.934			
	4559.312			
37.5	6802.719	6812.243	8.27243	0.12143
	6816.37			
	6817.64			

Repeatability data

Sr. no.	**Miconazole (MIC) 5 µg/ml**	**Ornidazole (ORN) 25 µg/ml**
1	4034.591	4559.521
2	4042.68	4568.662
3	4050.77	4564.741
4	4038.63	4564.082
5	4046.71	4573.218
6	4054.803	4581.133
Mean Peak Area	4044.697	4568.5595
SD	7.56403	7.695823042
% RSD	0.187011	0.168451851

Table 4: LOD and LOQ of MIC and ORN

Drug	**LOD [µg /ml]**	**LOQ [µg /ml]**
MIC	0.406	1.231
ORN	1.828	5.542

Table 5: Robustness study for MIC and ORN

Sr. No.	**Area at M.P +2**		**Area at M.P -2**		**Area at pH +2**		**Area at pH -2**	
	MIC	ORN	MIC	ORN	MIC	ORN	MIC	ORN
1	3925	4435	4131	4669	3852	4353	4127	4664
2	3949	4459	4155	4696	3876	4380	4151	4692
3	3977	4489	4180	4714	3900	4403	4176	4713

Avg. area	3950	4461	4156	4693	3876	4379	4151	4690
% RSD	0.6629	0.6012	0.5883	0.4829	0.62399	0.57866	0.58837	0.52108

Table: 6. System suitability data for the developed method

SYSTEM SUITABILITY PARAMETER	MIC	ORN
Retention time (min)	6.563± (0.239)minute	3.280 ± (0.381) minute
Resolution	12.202 ±(0.025)	
Asymmetric	1.578 ± (0.0093)	1.680 ± (0.0025)
Theoretical Plates	12031.15 ± (91.43)	13215.21 ± (41.71)

Table: 7. Assay of marketed formulation

Drug	Label Claim	Amount Found^{n} (µg)	%MIC ^{n} ± SD	%ONR ^{n} ± SD
MIC	100	104.15	104.585 ± 0.581	94.696 ± 0.498
ONR	500	473.82		

Table 8: Stability data

Stress condition	Miconazole (MIC)				Ornidazole (ORN)			
	Area	% deg Std	Area	% deg Samp	Area	% deg Std	Area	% deg Samp
Alkaline hydrolysis	3092.034	25.14	3124.597	24.35	3331.138	28.16	3293.079	28.98
Acidic hydrolysis	3496.781	15.34	3536.327	14.38	3537.475	23.71	3514.56	24.21
Oxidative Deg.	2941.445	28.78	2910.537	29.53	3038.669	34.47	3037.498	34.50
Dry heat	3070.441	25.66	3037.587	26.46	3723.532	19.70	3776.796	18.55
Photostab.	2770.557	32.92	2678.877	35.14	3723.546	19.70	3819.201	17.64

MIC Area of standard: 4637.109
ORN Area of standard: 4130.606

Table 9: Summary of validation parameters

PARAMETERS	MICONAZOLE	ORNIDAZOLE
Linear Range	2.5-7.5 µg/ ml	12.5-37.5 µg/ml
Regression Coefficient	0.9985	0.9988
Recovery %	100.18 % - 100.20 %	99.87 %- 99.90%
Repeatability (RSD, n=6)	0.1870	0.1684
Precision (RSD) Intra - day (n=3) Inter - day (n=3)	 0.20-0.27% 0.19-0.20%	 0.12–0.15% 0.11–0.15%
Limit of Detection (µg/ml)	0.40638536	1.828978073
Limit of Quantitation (µg/ml)	1.231470788	5.542357798
Robustness	Robust	Robust
Specificity	Specific	Specific

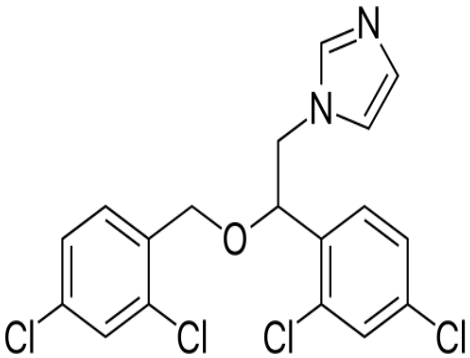

Fig: 1. Structure of Miconazole

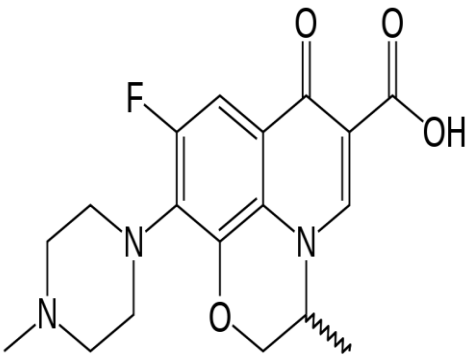

Fig: 2. Structure of Ornidazole

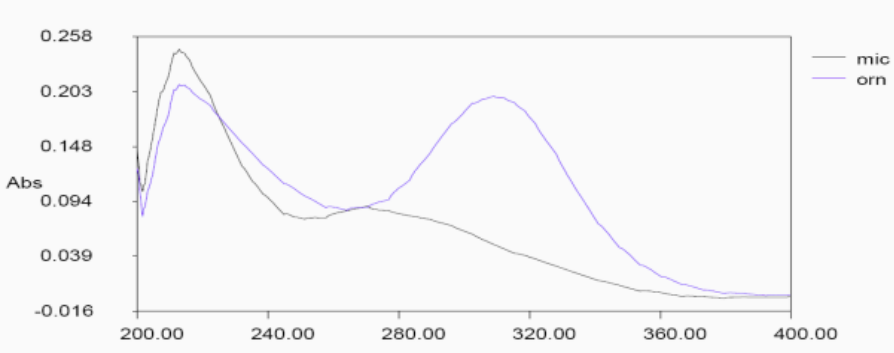

Figure 3: Selection of analytical wavelength

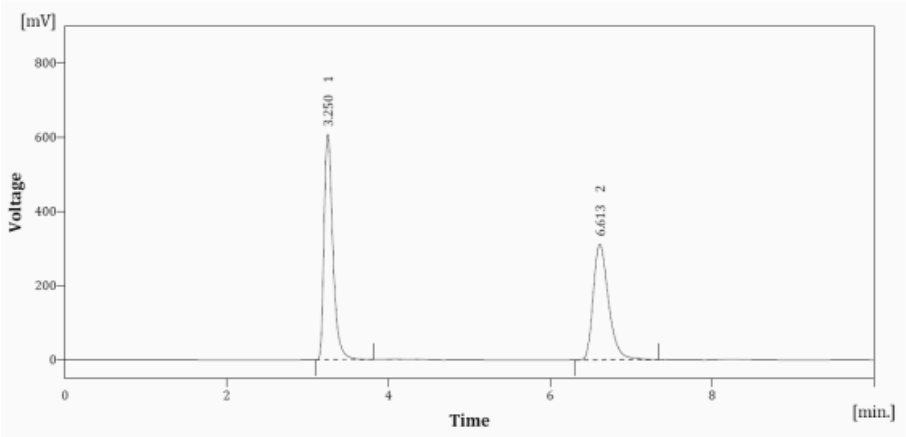

Figure 4: Chromatogram in optimized mobile phase

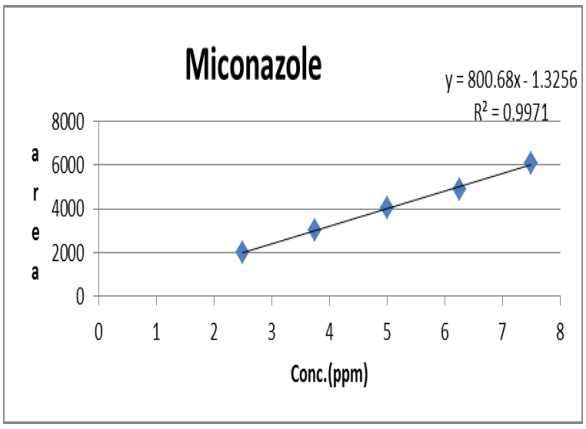

Fig: 5. Calibration curve of MIC

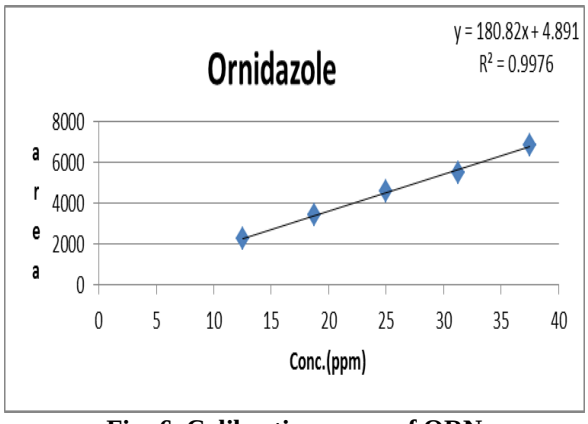

Fig: 6. Calibration curve of ORN

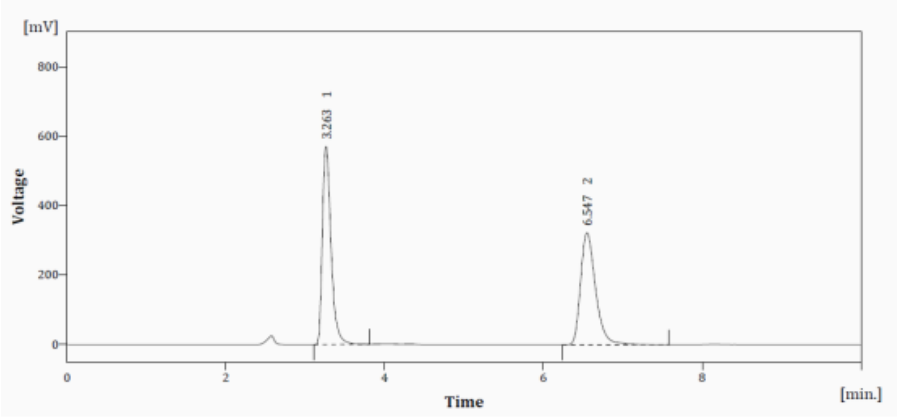

Fig: 7. Chromatogram of Miconazole nitrate (MIC) and Ornidazole (ORN) in dosage form

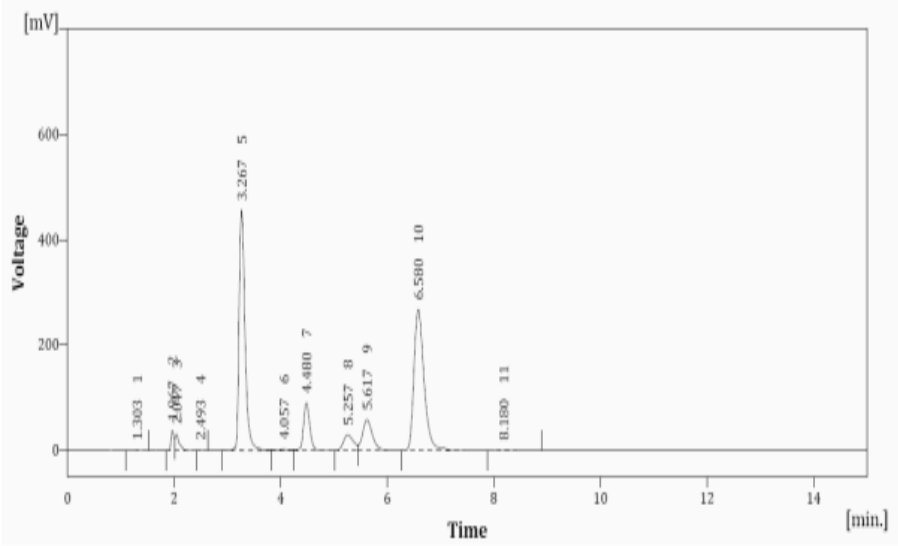

Fig: 8. Chromatograph of acid degradation study

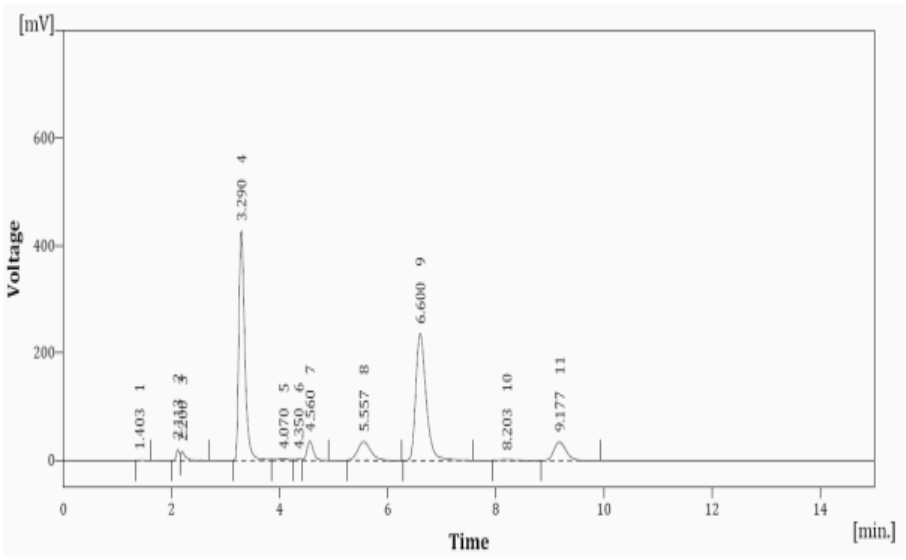

Fig: 9 Chromatograph of base degradation study

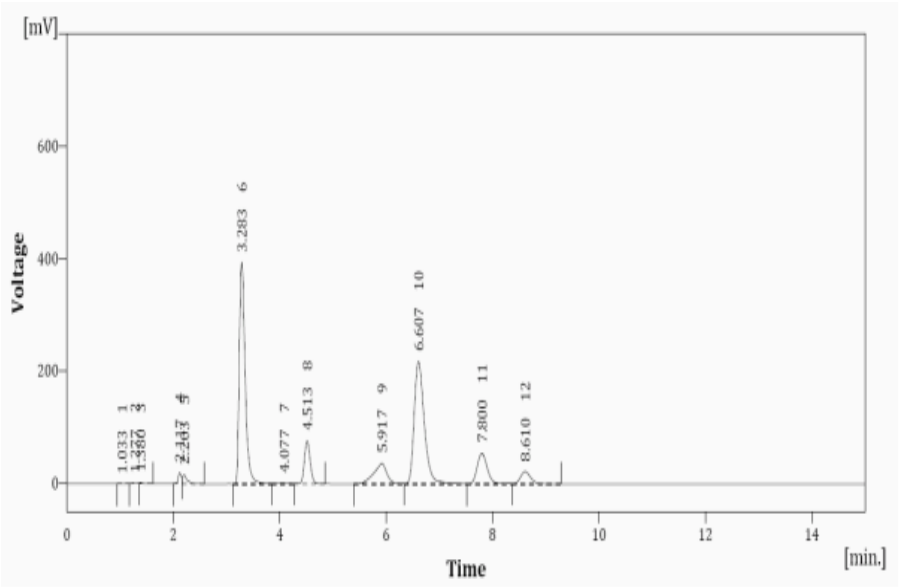

Fig: 10. Chromatograph of Oxidative degradation

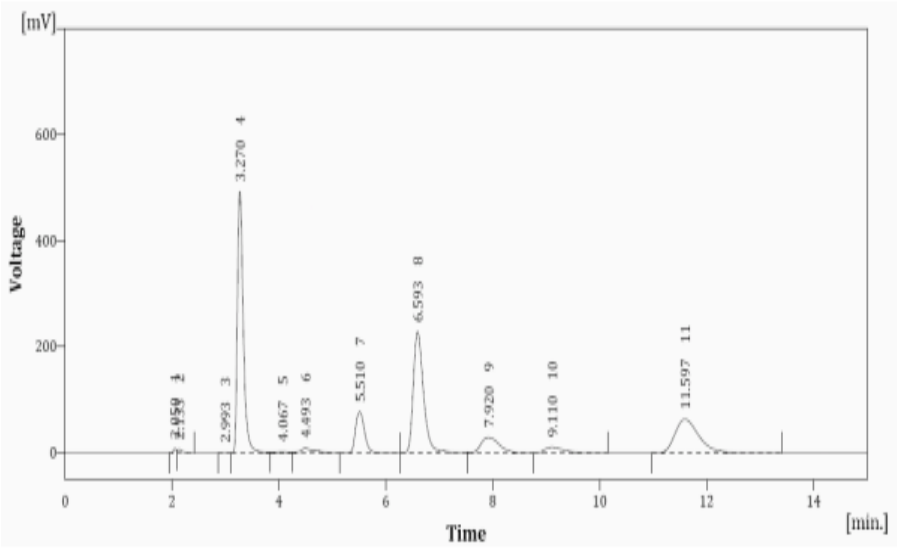

Fig 11: Chromatogram of Thermal degradation

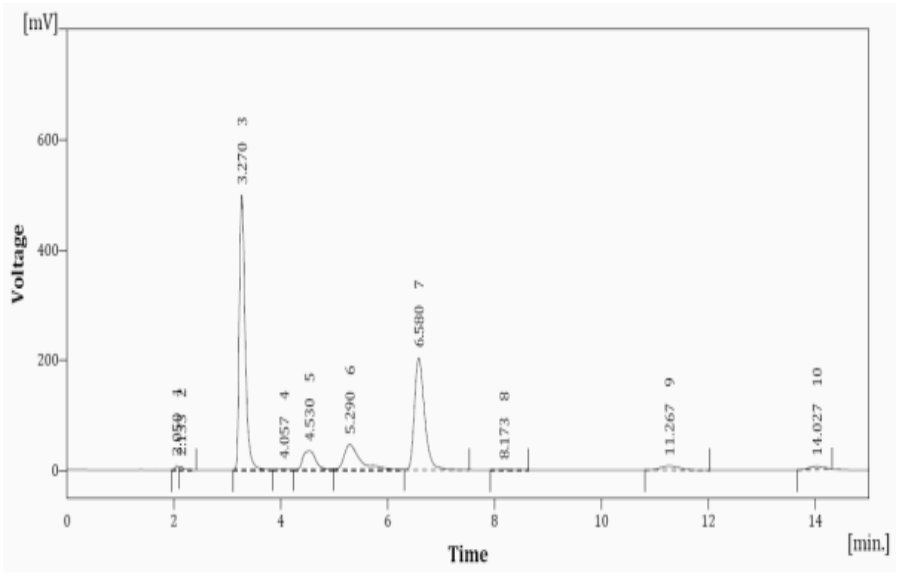

Fig 12: Chromatogram of Photolytic degradation

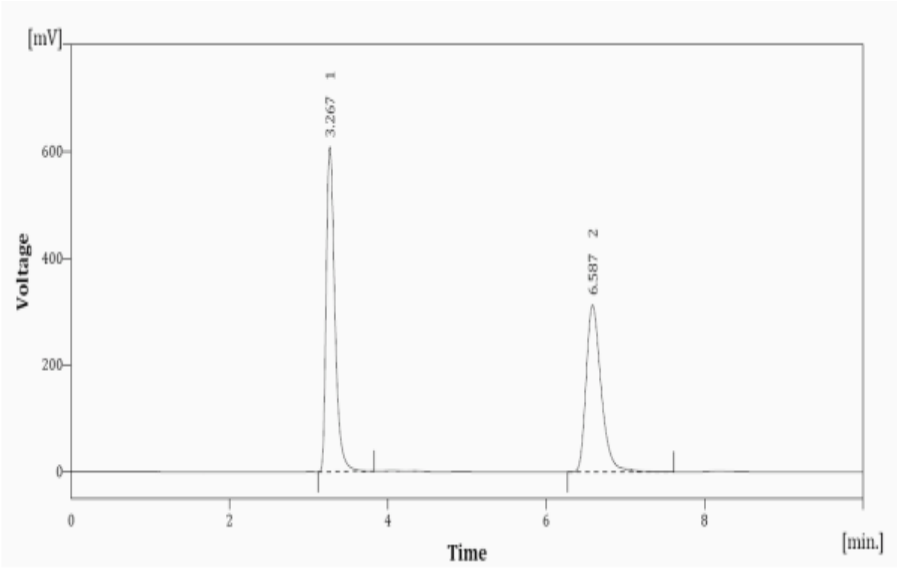

Fig 13: Chromatogram of control sample for stability

Conflict of Interest: None.
Ethical Permission: None**.**

Abbreviations
MIC- Miconazole
ORN- Ornidazole
µg- microgram

CONCLUSION

- RP-HPLC method was developed using C_{18} (250 x 4.6mm, 5µm) column as a stationary phase and buffer (0.05M KH_2 PO_4, pH 3.5): Methanol in the ratio of 25:75 as mobile phase. The flow rate was maintained at 1 ml/ min and detection was carried out at 236 nm where miconazole and ornidazole have significant absorbance. The retention times of miconazole and ornidazole were 6.58 and 3.26 min respectively. RP-HPLC method is linear in the concentration range of 2.5-7.5 µg/ ml miconazole and 12.5-37.5 µg/ml ornidazole with correlation coefficient found to be 0.9985 for miconazole and 0.9988 for ornidazole. The recovery was in the range of 100.18% - 100.20% for miconazole and 99.87 %-99.90% for ornidazole. Limit of detection for miconazole and ornidazole was found to be 0.40 and 1.82 respectively. Limit of quantification for miconazole and ornidazole was found to be 1.23 and 5.54 µg/ml respectively. The method was found to be accurate, precise, specific, selective, repeatable, robust and reproducible. Forced degradation studies were carried out and degradation product peaks were well resolved from drug peaks. In stress study it was found that miconazole and ornidazole were degraded in alkali medium, acidic medium and oxidative stress condition where as in other stress condition slightly degraded. The method was validated and found to be sensitive, accurate and precise and stability indicating.
- The developed stability indicating RP-HPLC methods were validated for linearity, accuracy, method precision, selectivity, sensitivity and robustness. It was found to be simple, sensitive, accurate, precise and robust.
- The mean percentage assay for Miconazole nitrate (MIC) and Ornidazole (ORN) in tablet was found to be 104.58 % and 99.69% respectively.
- These developed RP-HPLC method can be used for routine analysis of miconazole and ornidazole in bulk and their pharmaceutical formulations.

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