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Development and Validation of Stability Indicating Assay Method For Simultaneous Estimation of Azithromycin, Fluconazole and Ornidazole In Bulk and Its Dosage Form by RP-HPLC

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ABSTRACT

The present study was designed to develop simple accurate, precise, reproducible and validating of a stability indicating reverse phase high performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of azithromycin, ornidazole and fluconazole in bulk and its pharmaceutical dosage forms. Chromatographic separation of the three drugs was performed on a Phenomenex C_{18} column (250X4.6mm 5µm) as stationary phase with a mobile phase comprising of 20mM potassium dihydrogen phosphate : Acetonitrile (pH 4.8) in the ratio 30:70% v/v at a flow rate of 1ml/min and peak monitored at 254nm using PDA detector. The retention time of azithromycin, fluconazole, ornidazole and procaine hydrochloride (internal standard) was Rt1-2.8, Rt2-5.0, Rt3-6.3 and Rt-3.8minutes respectively. The linearity of azithromycin, fluconazole and ornidazole were in the range of 20-100µg/ml, 3-15µg/ml and 15-75µg/ml with an internal standard, procaine hydrochloride 5µg/ml respectively. The accuracy of the method was found to be 98-102% and %RSD was found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. The limit of detection for azithromycin, fluconazole and ornidazole was found to be 0.34, 2.80 and 0.76µg/ml respectively whereas, the limit of quantification was found to be 1.05, 8.6 and 2.31µg/ml respectively. Forced degradation studies were conducted to know the stability of the drug samples under various stress conditions like acid, base, peroxide and photolytic degradation according to ICH guidelines. Results are validated statistically as per ICH guidelines.

Keywords: RP-HPLC, Azithromycin, fluconazole, ornidazole estimation, Forced degradation study, validation.

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INTRODUCTION

Drugs with antifungal and antiprotozoal activity have been used in the treatment of the same. In many cases, drugs with two active ingredients are prescribed to the patients to have an added advantage. Many of these antibacterial drugs are found in combination with antifungal and antiprotozoal drugs which are highly effective against fungal and protozoal infections. Azithromycin(2R,3S,4R,5R,8R,10R11R,12S,13R,14R)-13-[2,b-dideoxy-3-c-methyl-α-L ribohexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6-8,10,12,14-heptamethyl-11-{[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy}-1-oxa-6-azacyclopentadecan-15-one used as an antibacterial drug are shown in Figure.1. Fluconazole 2-(2,4 – diflucophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)propan-2-ol used as an antifungal drug are shown in Figure.2. Ornidazole (RS)-1-chloro-3-(2-methyl-5-Notroimidazole-1-yl) propan-2-ol used as an antiprotozoal drug are shown in Figure.3. It is highly effective for bacterial and protozoal infections and is available in the tablet form (combi-kit).Forced degradation studies are used to identify reactions which may occur to degrade a processed product. Stability testing provides information about how the quality of bulk drug varies with time under the influence of various environmental factors such as temperature, humidity and light. Stability is the most important criteria in pharmaceutical formulation, the drug which is free from its degraded product is safe and effective for patients with this background as study has been undertaken to develop a stability indicating RP-HPLC method. Survey of literature revealed that few analytical method have been developed for the determination of Azithromycin, Fluconazole and Ornidazole individually. Hence, an attempt has been made to develop a simple, accurate, precise and reproducible RP-HPLC method for stability indicating simultaneous estimation of azithromycin, ornidazole and fluconazole in bulk and its pharmaceutical dosage forms.

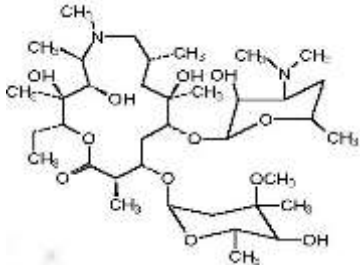

Figure 1: Azithromycin

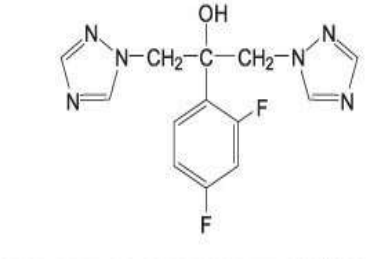

Figure 2: Fluconazole

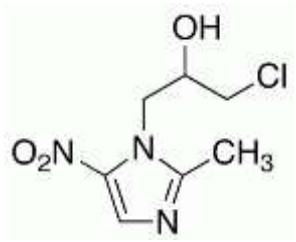

Figure 3: Ornidazole

MATERIALS AND METHOD

Standard bulk drug sample azithromycin was supplied by Pharmafabrikon, Madurai Tamilnadu, India as gift sample. Fluconazole and ornidazole obtained from yarrow chem. Products, Mumbai, India. Tablets of combi-kit (orflaz - kit) were procured from the local market. All other reagents used were of HPLC grade.

The chromatographic separation was performed on a shimadzu high performance liquid chromatographic instrument and equipped with a Phenomenex C_{18} (250X4.6mm, 5µm) column, LC 10AT VP series pump for solvent delivery and variable wavelength programmable SPD-M-10 AVP PDA detector. Data was analyzed by using lab solution software. The mobile phase and all the solutions were filtered through 0.45mm degassed by ultrasonication.

Preparation of mobile phase

The mobile phase was Acetonitrile and potassium dihydrogen phosphate buffer (pH 4.8) – ratio of 70:30% v/v. The mobile phase was filtered through a 0.45µm millipore filter and degassed by sonication for 15minutes.

Preparation of stock and standard solution

Each drug of 10mg of azithromycin, fluconazole and ornidazole was taken in a separate 10ml volumetric flask up to 10ml with methanol. 5ml from stock solution were taken and transferred into 50ml volumetric flask and made up to 50ml with mobile phase to get a concentration of 100µg/ml. subsequent dilutions of this solution were made with mobile phase to get a concentrations of 20-100µg/ml, 3-15µg/ml and 15-75µg/ml and were mixed with an internal standard, procaine hydrochloride 5µg/ml.

Preparation of sample solution

For the analysis of tablet dosage form, 3 tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solution of azithromycin (1000µg/ml), fluconazole (150µg/ml) and ornidazole (750µg/ml) along with an internal standard procaine hydrochloride 5µg/ml were prepared by dissolving average of tablets; equivalent to 1000mg of AZI, 150mg of FLU and

750mg of ORNI and made up to 50ml with mobile phase sonicated for 15min and later filtered the solution 0.45µm filter.

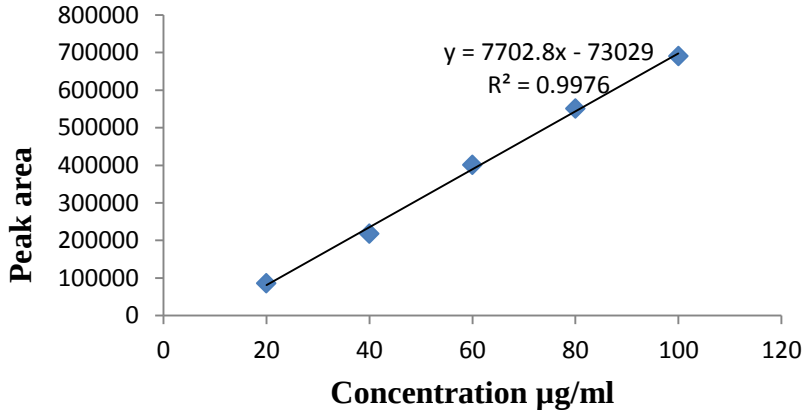

Figure 4: Calibration curve of Azithromycin

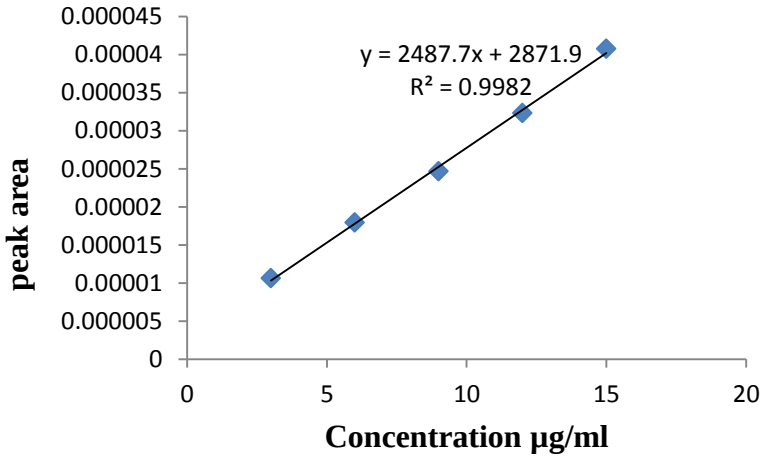

Figure 5: Calibration curve of Fluconazole

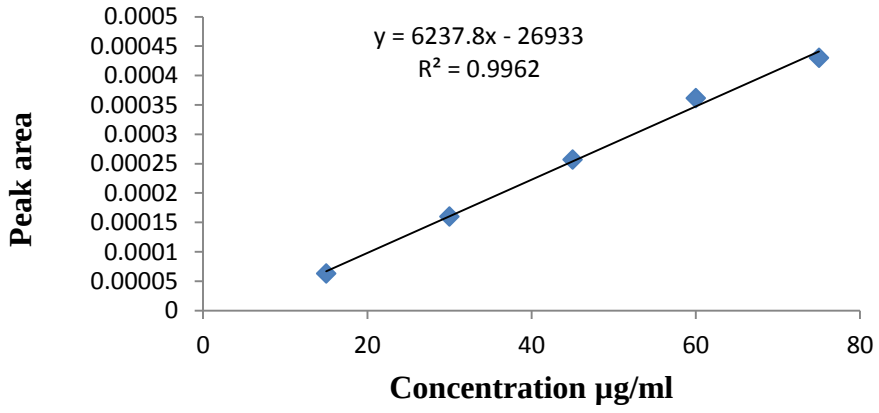

Figure 6: Calibration curve of Ornidazole

Optimum chromatographic condition

Proper selection of chromatographic conditions can be achieved by studying various factors which affect the studies such as effect of pH, stationary phase, ratio of mobile phase and flow rate. The separation was performed on an analytical Phenomenex C_{18} 5m particle size (250X4.6mm) column as stationary phase. The mobile phase consist of 20mM potassium dihydrogen phosphate: Acetonitrile (pH 4.8) in the ratio 70:30% v/v at a flow rate of mobile phase 1ml/min and detector wavelength was set at 254nm. The mobile phase was filtered through 0.45 Millipore filter in glass apparatus and degassed by ultrasonication. The retention time of azithromycin, fluconazole, ornidazole and procaine hydrochloride (Internal standard) was Rt1-2.8, Rt2-5.0, Rt3-6.3 and Rt-3.8minutes respectively Figure7.

FORCED DEGRADATION STUDY

Forced degradation of each drug substances was carried out under thermolytic, photolytic, acid/base hydrolytic and oxidative stress conditions. Thermal and photo degradation study was carried out in solid state. Solutions were prepared by dissolving drug substance in small volume of methanol and later diluted with Hydrochloric acid, 0.1N sodium hydroxide or hydrogen peroxide to achieve a concentration of 100µg/ml each of AZI, FLU and ORNI. After the degradation substance were diluted with mobile phase to achieve a concentration each sample of 10µg/ml of AZI, FLU and ORNI respectively.

After degradation treatment was completed, the stress content was allowed to equlibriate at room temparature and diluted with mobile phase to attain (100µg/ml of AZI, 75µg/ml of ORNI and 15µg/ml of FLU) this concentration (Table 8-11). The specific degradation condition as follows,

1. Thermal study

   For thermal study, sample of drug substances were placed in a controlled temperature oven at 60ºC for 72 hours.

2. Acid hydrolytic stress testing

   To 1ml of stock solution Azithromycin, ornidazole and fluconazole, 1ml of 0.1N hydrochloric acid was added and refluxed for 3hours at 60ºC.

3. Base hydrolytic stress testing

   To 1ml of stock solution Azithromycin, Ornidazole and Fluconazole, 1ml of 0.1N sodium hydroxide was added and refluxed for 3hours at 60ºC.

4. Oxidation study

   To 1ml of stock solution Azithromycin, Ornidazole and Fluconazole, 1ml of 30% hydrogen peroxide was added and refluxed for 3hours at 60ºC.

5. Photolytic degradation

In this study, the drug substance were exposed to direct sunlight for 5days to determine the effect of irradiation on the stability of the three drugs in solid state.

Method Validation

The method was validated as per ICH guidelines [20, 21] with respect to linearity, range, specificity, limit of detection and limit of quantification.

RESULTS AND DISCUSSION

System suitability

System suitability, an integral part of analytical procedures, based on concept that equipment, sample, electronics an integral system that can be evaluated. System suitability was assessed by six replicate of the mixture containing 10g/ml of both the drugs. The resolution, number of theoretical plate and tailing factor were calculated. Table 1.

Table 1: System suitability data for developed method

S.No	Parameters	Azithromycin	Fluconazole	Ornidazole
1	Linearity range(µg/ml)	20-100	3-15	15-75
2	Correlation coefficient (R^2)	0.9976	0.9982	0.9962
3	Intercept	73029	2871.9	26933
4	Slope	7702.8	2487.7	6237.8
5	Resolution	-	6.05	4.12
6	Theoretical plates	2428.5	4585	5662.4
7	Tailing factor	1.15	1.01	0.9

Linearity

The linearity of an analytical procedure is its ability to obtained test results which are directly proportional to the concentration of analyte in the sample. The plot of peak area Vs the respective concentration of AZI, FLU and ORNI were found to be linear in the concentration in the concentration range of 20-100µg/ml, 3-15µg/ml and 15-75µg/ml respectively. The results of linearity and regression equation for AZI, FLU and ORNI were given in figure. Correlation co-efficient were found to be 0.9976, 0.9964 and 0.9962 for AZI, FLU and ORNI. The standard curves for AZI, FLU and ORNI are shown in Figure.4, Figure.5 and Figure.6, respectively and data's are presented in Table 2.

Table 2: Linearity, LOD and LOQ data

Parameters	Azithromycin	Fluconazole	Ornidazole
Correlation coefficient(R^2)	0.9976	0.9982	0.9962
Slope	7702.8	2487.7	6237.8
Intercept	73029	2871.9	26933
LOD(µg/ml)	0.348	2.80	0.76

LOQ(µg/ml)	1.05	8.6	2.31

Accuracy

The accuracy of the method assay determination was achieved at three concentrations levels of 50%, 100% and 150% for AZI, FLU and ORNI. Known amount of standard drug concentration was added to the sample and peak area was determined. The contents were determined from the respective chromatogram. Table 3.

Table 3: Percentage Recovery report

Level	%Recovery± SD			%RSD		
	AZI	FLU	ORNI	AZI	FLU	ORNI
50%	98.90±0.10	98.93±0.24	98.72±0.25	0.102	0.243	0.257
100%	99.02±0.37	99.08±0.22	99.29±0.19	0.381	0.230	0.199
150%	98.81±0.33	99.05±0.33	98.80±0.18	0.337	0.337	0.184

RSD Relative standard deviation; SD Standard deviation

Precision

Precision of the method was studied as intraday and interday variations and also as repeatability, intraday precision was determined by triplicate analysis of each solution on single day. For interday validation, concentrations were determined on three separate days. The %RSD values less than 2.0% indicate that the method was precise. Table 4, 5 shows the precision data for the method.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of component which may be expected to be present. There was no other interfering peak around the retention time of AZI, FLU and ORNI and also the base line did not show any significant noise.

Sensitivity

The sensitivity of the method was determined by establishing the limit of detection and limit of quantification, which was calculated by the method based on standard deviation of intercept and slope(s) of the calibration.

The LOD and LOQ results were displayed in table 2.

Table 4: Precision studies for (intraday) Azithromycin, fluconazole and Ornidazole

S.No	Statistical data	Azithromycin	Fluconazole	Ornidazole
1	% Mean	99.98%	99.94%	100.04%
2	Standard deviation	2224.7	112.38	1536.4
3	%RSD	0.604	0.464	0.578

RSD Relative standard deviation

Table 5: Precision studies for (interday) Azithromycin, fluconazole and Ornidazole

S.No	Statistical data	Azithromycin	Fluconazole	Ornidazole
1	% Mean	98.19%	98.54%	100.29%
2	Standard deviation	4855.05	176.6	2172
3	%RSD	1.07	0.68	0.83

[RSD] Relative standard deviation

Table 6: Assay report for tablet formulation

Drug	Amount labeled(mg.ml)	Amount found(mg/ml)	%Assay	%RSD
Azithromycin	1000	991	99.10	0.431
Fluconazole	150	149.9	98.98	0.203
Ornidazole	750	742.25	99	0.612

[RSD] Relative standard deviation; [SD] Standard deviation

5.7. Robustness

This parameter was carried out to check the ability of the system to give unaffected results for small deliberate changes in system parameters and method parameters. The robustness of the method was evaluated by varying the three of the chromatographic conditions those are mobile phase composition, pH and flow rate of the pump.

Analysis of formulation

Formulation used for tablet and brand name orflaz-kit. 3 tablets were weighed and taken into a mortar, crushed and then uniformly mixed. Test stock solution of azithromycin(1000µg/ml), ornidazole(750µg/ml) and fluconazole(150µg/ml) were prepared by dissolving average of tablets; equivalent to 1000mg of AZI, 750mg of ORNI and 150mg of FLU and made up to 50ml with mobile phase sonicated for 15min and later filtered the solution 0.45 micron syringe filter (figure 8). The percentage assay for formulation results were displayed in Table 6.

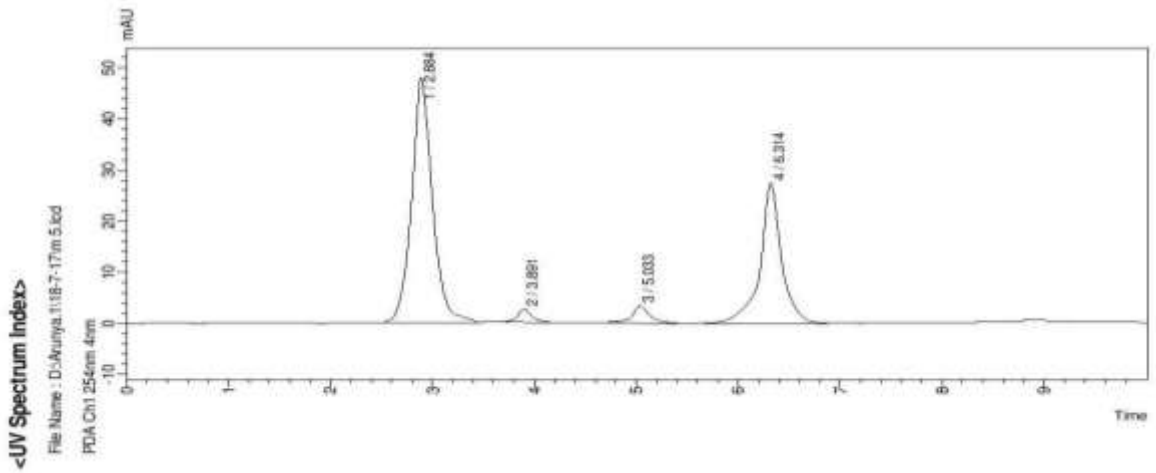

Figure 7: Chromatogram of Azithromycin (Rt 2.8), Fluconazole (Rt 5.0) and Ornidazole (Rt 6.3) and Procaine hydrochloride (Rt 3.8) – (Internal standard).

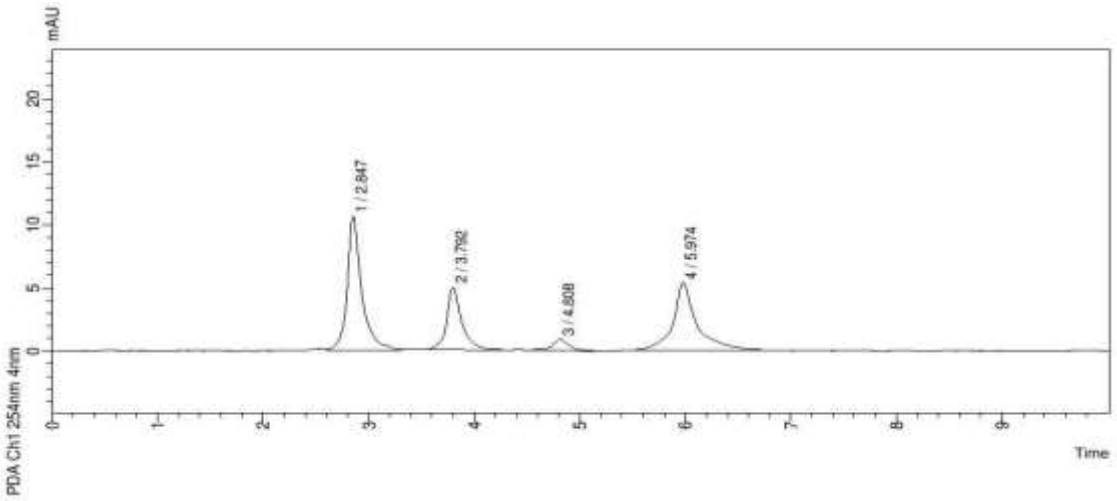

Figure 8: Chromatogram of formulation

Forced degradation studies

The study involves acid hydrolysis (1ml 0.1M HCL heated for 3hours at 60ºC) alkali hydrolysis (1ml 0.1M NaOH heated for 3hours at 60ºC) oxidative degradation (30% H_2O_2 heated for 3hours at 60ºC) and thermal degradation (sample placed in oven 60ºC for 72hours). For photolytic stress studies, sample was exposed to direct sun light for 5 days. Degradation results were displayed in Table 7, 8, 9 and 10 respectively.

Table 7: Degradation studies for Azithromycin

Stress condition	Sample concentration	Area	%Assay	%Degradation
Acid	10µg/ml	62406	90	9.54
Base	10µg/ml	64270	93	6.8
Oxidation	10µg/ml	64037	92	7.17
Photolytic	10µg/ml	64115	92	7.06
Thermal	10µg/ml	62569	90	9.3

Table 8: Degradation studies for Fluconazole

Stress condition	Sample concentration	Area	%Assay	%Degradation
Acid	10µg/ml	24296	89	10.05
Base	10µg/ml	24316	90	9.97
Oxidation	10µg/ml	24569	90	9.04
Photolytic	10µg/ml	25621	94	5.14
Thermal	10µg/ml	24593	91	8.95

Table 9: Degradation studies for Ornidazole

Stress condition	Sample concentration	Area	%Assay	%Degradation

Acid	10µg/ml	49057	91	8.64
Base	10µg/ml	50452	93	6.04
Oxidation	10µg/ml	49773	92	7.30
Photolytic	10µg/ml	49326	91	8.14
Thermal	10µg/ml	48264	89	10.11

Table 7: Degradation of Mixture

Stress condition	**Sample concentration**	**%Assay**			**%Degradation**		
		AZI	FLU	ORNI	AZI	FLU	ORNI
Acid	100µg/ml	92	97	94	7.80	2.43	5.11
Base	100µg/ml	95	94	91	4.05	5.69	9.02
Oxidation	100µg/ml	93	95	93	6.32	4.62	6.84
Photolytic	100µg/ml	90	93	95	9.01	6.43	4.62
Thermal	100µg/ml	94	90	93	5.91	9.50	6.30

CONCLUSION

From the discussion a simple, precise and novel stability indicating RP-HPLC method has been developed and validated for the determination of Azithromycin, Fluconazole and Ornidazole. The method was primarily designed for assay of Azithromycin, Fluconazole and Ornidazole in Tablet. Moreover, the content of degradation of Azithromycin, fluconazole and Ornidazole in various conditions such as Acidic, basic, oxidation, photolytic and thermal degradation, were observed and quantitatively analyzed by this HPLC method. The application of this type of estimation in the selected drug formulation form was proved.

The present study helps in identifying the degraded products of Azithromycin, Fluconazole and Ornidazole in bulk during their storage conditions and transport condition. This research work is to report its stability studies with degraded product identification, which is helpful for determining the toxicity of the degraded product and also caution to the storage condition.

The study concluded that developed method is statistically significant and the result of validation parameters it is evident that the proposed method is accurate, precise, selective, specific, robust and stability indicating. The good percentage of recovery indicates the reproducibility and accuracy of the method. Hence, this developed analytical method can be used in the industry for the routine analysis with more accurate results.

Different mobile phase were tried to select the ideal mobile phase. Among these Acetonitrile: Potassium dihydrogen phosphate buffer (70:30) was found to be ideal, since it gave good resolution and peak symmetry. The flow rate was found to be optimized at 1ml/min. Detection was carried out at 254nm by PDA detection. The linearity of Azithromycin 20-100µg/ml, Fluconazole

3-15µg/ml and Ornidazole 15-75µg/ml, the correlation coefficient was 0.997, 0.998 and 0.996 respectively.

The degradation study results shows that the drugs were stable at acidic, alkaline and oxidation and also showed liability in dry heat at 60ºC and photolytic condition.

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