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Method Development and Validation of Fluconazole and Ivermectin in pure and Combined Dosage form using UV by Q- absorption ratio and Vierotd's method

R. Swetha Sri*, Goli Sowmya, Mogili Sumakanth

Department of pharmaceutical analysis, RBVRR women's college of pharmacy, Barkatpura, Hyderabad. India.

Abstract

Multicomponent analysis involves simultaneous estimation of drug substances in combined dosage form. A comparative study between Q-Absorption ratio technique and Simultaneous Equation Method has been established for effective implication of the developed method. Numerous trials were performed as a part of solvent selection, both the drugs under study Fluconazole (FLN) and Ivermectin (IVR) have shown good solubility in methanol (Spectroscopic Grade). The absorption maxima (λmax) were found to be 261nm for FLU and 245nm for IVR respectively. Both the drugs were showing same extinction coefficient (Isosbestic point) at 261nm. A comparative study is proposed to be established for simultaneous equation and Q-absorption ratio method. Between the concentration and absorbance, calibration curves revealed a linear relationship. The regression line equation was established and identified r^2 for Fluconazole and Ivermectin is 0.999 and 0.999. Fluconazole and Ivermectin were assessed by the validated method when each of the drug individually subjected to various stress conditions like concentrated acidic, basic, peroxide, excessive light and thermal. For estimation, the suggested approach was used for drug content in locally available marketed formulations which has proven successful for routine analysis of the same with the application of vierotd's as well as Q-absorption method.

Keywords: Q-Absorption ratio method, Simultaneous equation method, analytical method validation, LOD, LOQ, Fluconazole, Ivermectin

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*Corresponding author

1. Introduction

Combination of fluconazole and ivermectin is used to treat, manage, prevent, and improve fungus infections, parasitic infection of skin and hair, Inflammatory diseases. (1-5) Chemically known Fluconazole as 2-(2,4-Difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl) propan-2-ol and Ivermectin as 22,23-dihydroavermectin B_{1a}+ 22,23-dihydroavermectin B_{1b}. Triazole antifungal fluconazole as viewed in Figure 1, is used to treat candidiasis and other fungi infections. It is commonly known as Diflucan used for treatment of both systemic and superficial fungal infections in a variety of tissues and also which stops the growth of fungi by preventing them from forming their own protective covering. (6-11) The FDA initially gave its permission to it in 1990. Itraconazole and ketoconazole are members of the same medication family as this azole antifungal. Among its many advantages over other antifungal medications, fluconazole can be taken orally. (12-16) Ivermectin, Chemical moeity given in Figure 2, an antiparasitic drug, paralyses and kills parasitic worms by attaching to their muscle and nerve cells. Ivermectin is a semi-synthetic antiparasitic drug that is produced from a class of very effective broad-spectrum antiparasitic compounds known as avermectins. (17-25) These agents were discovered in the fermentation byproducts of Streptomyces avermitilis. Ivermectin, also known as 22,23-dihydroavermectin B1a, is a compound consisting of two avermectins (26-28), with roughly 90% of it being 5-O-demethyl-22,23-dihydroavermectin A1a and 10% being 5-O-demethyl-25-de (1-methylpropyl) Avermect, 22,23-dihydro, 25-(1-methylethyl) (22,23-dihydro-avermectin B1b). Fluconazole and Ivermectin are estimated by two methods they are one employs the simultaneous equation approach, while the other the Q-absorption ratio approach. (29-33) Fluconazole and Ivermectin were estimated using the simultaneous equation approach at 261nm (λmax) and 245 (λmax) respectively. Precision, accuracy, linearity, LOD, LOQ, and specificity were all done as validation parameters, and all of the parameters were found to be within limits. The estimation of Fluconazole and Ivermectin in

combination and pure dose forms using projected approaches is said to be accurate and successful. (34,35)

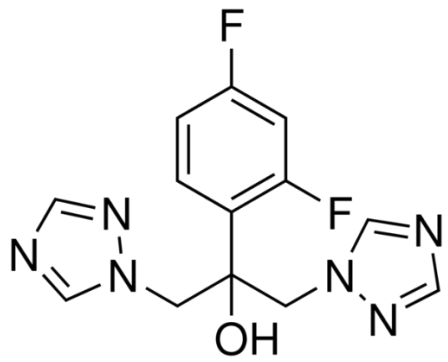

Figure 1. Chemical structure of Fluconazole

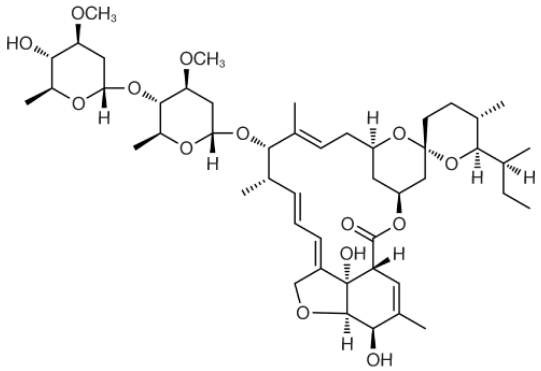

Figure 2. Chemical structure of Ivermectin

Simultaneous equation method: The concentrations in the samples were obtained using the formula below.

CX= A1ay2 – A2ay1 / ax1ay2 – ax2ay1

CY = A1ax2 – A2ax1 / ay1ax2 – ay2ax1

Cx and Cy are concentrations of FLU and IVR, respectively,

A1 and A2 are the mixture's absorbances at 261 nm and 245 nm, respectively

Similarly, ax1 and ax2 are the absorptivity of FLU at 261 nm and 245 nm, respectively.

ay1 and ay2 are absorptivity of IVR at 261nm and 245nm respectively,

Q-Analysis/Isobestic point method: The following equations were used to calculate concentrations in the samples.

Cx = (Qm-Qy / Qx-Qy). A1/ax1

Cy = (Qm-Qx / Qy-Qx). A2/ay1

Qm=A2/A1

Qx=ax2/ax1

Qy=ay2/ay1

Where, A1 & A2 are mixture absorbance at 245 nm & isobestic point, respectively.

Fluconazole and Ivermectin have different absorptivities at 245 nm.

Fluconazole & Ivermectin absorptions at the isobestic site are represented by ax2 and ay2.

2. Materials and methods

Instrument and Apparatus: All measures of absorbance for the studies were performed using an ELICO SL 210 UV and visible recording spectrophotometer with two matching 10-20 mm quartz cells. All weights were taken on Weighing Machine (Contech), Pipettes, Burettes and Beaker (Borosill) were used.

Chemicals and Reagents: Pure FLU and IVR kindly provided by Aurobindo Pharma with 99.5% purity as referred by the supplier. The formulation NUFORCE-PLUS (Fluconazole and Ivermectin 150:6).The pure form of both the drugs FLU and IVR has shown good solubility in methanol, it has been used as a diluent.

Experimental:

Fluconazole

Standard stock solution preparation: Standard Stock was made by combining 10 mg with 10 ml of diluent to make 1000µg/ml

Preparation of Working Standard solution: To achieve a concentration of 100 µg/ml, 1 ml of the standard stock solution was pipetted into a 10 ml volumetric flask. Pipetted 10 ml of the volumetric flask's working standard solution into 1 ml of it to achieve 10 µg/ml.

Ivermectin

Standard stock solution preparation: In order to create 1000µg/ml(ppm) of the Standard Stock, 10 mg were combined with 10 ml of diluent.

Preparation of Working Standard solution: Pipetted 1ml of 1000µg/mlof the diluent into a volumetric flask of 10 ml, then diluted the standard stock solution to the appropriate concentration 100µg/ml(ppm). Into a 10 ml volumetric flask, 1 ml of the working standard solution was pipetted. Then using the diluent, diluted it to the required concentration 10µg/ml(ppm).

Determination of λ_{max}: The max was determined by separately scanning the standard Concentration of FLU and IVR at various concentrations. As illustrated in Figure. 3, λ_{max} is determined between 200 and 400 nm, where the isosbestic point was discovered to be 260.7.

Calibration curve preparation: In order to obtain concentrations of 2.5–100µg/ml of FLU and 0.1–4µg/ml of IVR, the necessary aliquots of each medication were pipetted out of standard stock solutions into a series of 10 ml volumetric flasks, and the volume was topped off with methanol. For each drug, solutions of varying concentrations were examined at the appropriate wavelengths, and absorbance was noted.

Preparation of tablets for assay: The procedure was developed to incorporate the FLU and IVER formulation, which was marketed in doses of 150 mg and 6 mg of FLU and IVER, respectively. 20 tablets, on average, were taken and ground into a powder and then

transferred to volumetric flask which is 10ml. The volume of the flask has been then filled to the appropriate level with methanol (FLU 100µg/ml and IVR 10 µg/ml). Further dilutions were performed on the stock solutions to obtain the concentrations of 2.5 µg/ml & 1µg/ml of FLU and IVR, respectively. For the formulation, the same process as was described for the pure medication was used. Measurements of absorbance at 261 nm and 245 nm were used to determine the amounts of both FLU and IVR.

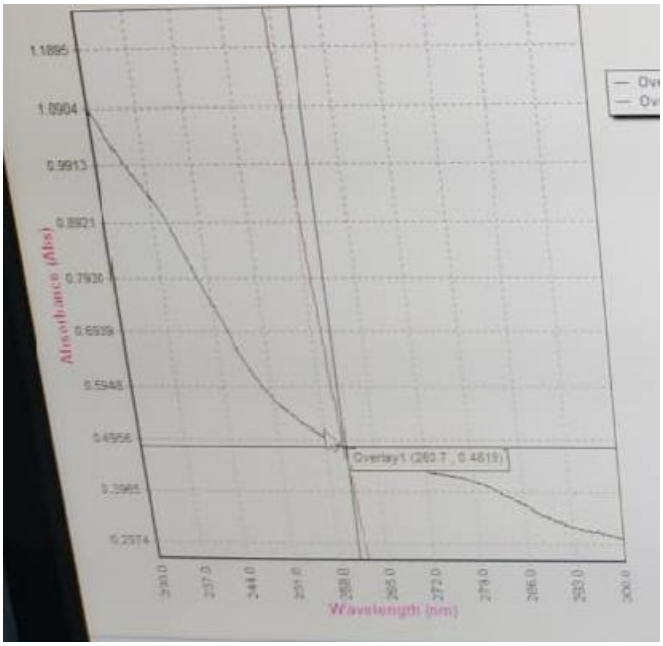

Figure 3. The Isobestic Point was established at the λmax 260.7 showing absorbance 0.4819

Simultaneous equation method

As the maximum absorbance of FLU & IVR individually using methanol, 261 nm and 245 nm, were chosen as the two wavelengths for the procedure. Standard stock solutions for both medicines were made separately in methanol at concentrations of 100µg/ml and 10µg/ml. To obtain a series of standard dilutions of 2.5-100 µg/ml for FLU and 0.1-4 µg/ml for IVR, the stock solutions of each medicine were separately further diluted with methanol. The absorbabilities (A 1%, 1 cm) for both medicines at both absorption maxima were calculated using the obtained absorbances at a chosen wavelength.

Q-analysis method

The wavelengths 245 nm and 260.7 nm were chosen for the Q-analysis approach because they correspond to the isobestic point in methanol and the maximal wavelength for IVER, respectively. Standard stock solutions were prepared separately in methanol at concentrations of 100µg/ml and 10µg/ml for each medication. To create a series of standard concentration-based solutions of 2.5–100µg/ml for FLU and 0.1–4µg/ml for IVR the stock solutions of each medication were diluted even further individually using diluting agent as methanol. The absorbtivities (A 1%, 1 cm) for both drugs at the two wavelengths were calculated based on the measured absorbances at the chosen wavelength.

Recovery Study

Recovery experiments were conducted in accordance with ICH recommendations to evaluate the suggested method's accuracy. Standard solutions of all two pharmaceuticals, corresponding to 50, 100, and 150% of their drug content, were added to the solutions that had already been analysed. Replicated studies were used to conduct recovery studies.

Analytical Method Validation

The analytical method was validated in accordance with ICH requirements for criteria such precision, accuracy, robustness, and recovery, linearity, limit of detection (LOD),limit of quantitation (LOQ). The calibration curve's least squares linear regression analysis was used to demonstrate linearity. By adding two different amounts of FLU and IVR to the placebo preparation (equivalent to Concentrations of 50%, 100%, and 150% for exam preparation), and then comparing the measured and real concentrations, accuracy was examined. Three solutions, each estimated twice, were created for each level. Test sample preparation was subjected to six independent analyses. were conducted, and the percentage RSD was calculated to assess the method's accuracy as well as intra-day repeatability. The method's intermediate (interday) accuracy was examined by having a different person follow the same steps under identical experimental settings on various days. The mathematical formulas

LOD = 3.3 x SD/S

LOQ = 10 x SD/S

were used to compute the LOD and LOQ of FLU and IVR.

By altering the UV analyst while maintaining the other conditions, the proposed method's robustness was tested (solvent, dilution, UV spectrophotometer).

Statistical Analysis: Means, standard deviations (SD), relative deviations from the mean (RSD), and linear

regression analysis were calculated using Microsoft Excel 2007.

3. Results and discussion

Precision: The % RSD values that were discovered to be less than 2 show that this method is accurate for determining the pure form were shown in Table 1.

Table 1. Precision results of Fluconazole & Ivermectin

Fluconazole			Ivermectin		
Concentration (µg/ml)	**Intra-day**	**Inter-day**	**Concentration (µg/ml)**	**Intra-day**	**Inter-day**
25	0.9407	0.9211	1	0.4751	0.4512
25	0.9412	0.9212	1	0.4741	0.4523
25	0.9421	0.9232	1	0.4732	0.4531
25	0.9435	0.9211	1	0.4737	0.4536
25	0.9462	0.9241	1	0.4761	0.4576
25	0.9465	0.9252	1	0.4782	0.4581
Average	0.9433	0.92265	Average	0.475067	0.454317
Standard deviation	0.002501	0.001778	Standard deviation	0.001853	0.002859
%RSD	0.265107	0.192758	%RSD	0.390111	0.629288

Linearity: By taking concentration at X-axis and absorbance at Y-axis data were used to plot a calibration curve as depicted in Table 2. as shown below against standard concentration from 2.5-100µg/ml for FLU and 1-10 µg/ml.The regression equation was Y=0.0569x+0.008 and Y=0.0916x-0.0327 for FLU and IVR, according to Figureures 4 and 5.Correlation coefficient was 0.9998 and 0.9991 for FLU and IVR.

Table 2. Linearity results of Fluconazole and Ivermectin

Fluconazole		Ivermectin	
Concentration(µg/ml)	**Absorbance**	**Concentration(µg/ml)**	**Absorbance**
2.5	0.0597	0.1	0.155
5	0.184	0.2	0.192
7.5	0.2877	0.3	0.238
10	0.3919	0.4	0.2742
12.5	0.4965	0.5	0.3112
15	0.5881	0.6	0.3431
17.5	0.6681	0.7	0.3575
20	0.7656	0.8	0.3812
22.5	0.8694	0.9	0.4221
25	0.9407	1	0.4751
50	1.959	2	0.7941
75	2.947	3	1.156
100	3.956	4	1.459

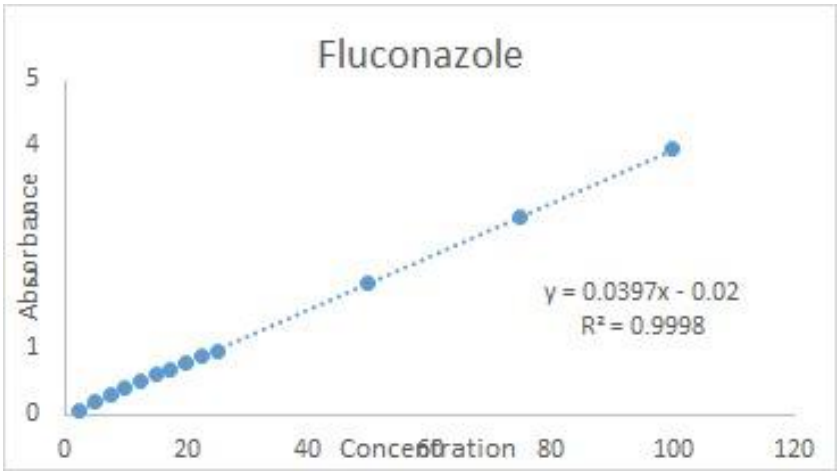

Figure 4. Calibration plot of Fluconazole

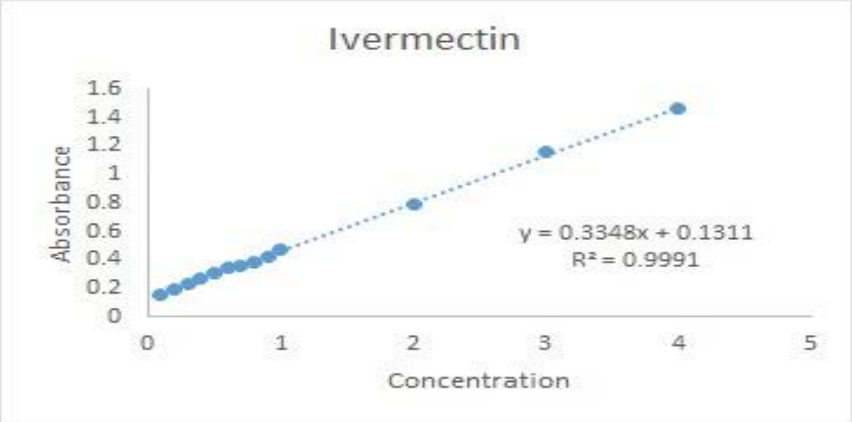

Figure 5. Calibration plot of Ivermectin

LOD & LOQ:

LOD values are 0.2078µg/ml and 0.0185µg/ml for FLU and IVR.

LOQ values are 0.6299µg/ml and 0.0553µg/ml for FLU and IVR.

Table 3. LOD &LOQ results of Fluconazole & Ivermectin

Drug	LOD	LOQ
Fluconazole	0.2078µg/ml	0.6299µg/ml
Ivermectin	0.0185µg/ml	0.0553µg/ml

Accuracy:

Recovery studies were carried out in three replicates of each concentration level, and the percent recovery was calculated, to determine accuracy. Recovery should range from 98 to 102%.

Table 4. Accuracy results of Fluconazole & Ivermectin

Fluconazole				**Ivermectin**			
%Level	**Absorbance**	**% Recovery**	**Mean% Recovery**	**%Level**	**Absorbance**	**% Recovery**	**Mean% Recovery**
50% (5ppm+15ppm)	0.6212 0.6391 0.6336	98.15% 98.51% 98.47%	98.15%	50% (0.2ppm+ 0.6ppm)	0.4112 0.4321 0.4221	99.51% 98.32% 98.77%	98.55%
100% (10ppm+15ppm)	0.8342 0.8821 0.8830	96.14% 98.84% 99.15%	99.59%	100% (0.4ppm+ 0.6ppm)	0.5142 0.5821 0.5830	98.14% 98.84% 99.15%	98.59%
150% (15ppm+15ppm)	1.1831 1.1184 1.1681	98.89% 99.14% 99.85%	99.84%	150% (0.6ppm+ 0.6ppm)	0.7831 0.7146 0.7671	97.85% 99.34% 99.61%	99.56%

Robustness: As with deliberate changes made in absorption maxima at its isosbestic point were illustrated in Table 5.

Table 5. Robustness results of Fluconazole & Ivermectin

Fluconazole				**Ivermectin**			
Concentration (µg/ml)	**260nm**	**261nm**	**262nm**	**Concentration (µg/ml)**	**244nm**	**245nm**	**246nm**
25	0.9130	0.9407	0.9821	1	0.4113	0.4512	0.4822
25	0.9140	0.9412	0.9898	1	0.4190	0.4523	0.4899
25	0.9152	0.9421	0.9812	1	0.4181	0.4531	0.4816
25	0.9112	0.9435	0.9835	1	0.4125	0.4536	0.4833
25	0.9151	0.9462	0.9831	1	0.4131	0.4576	0.4834
25	0.9111	0.9465	0.9843	1	0.4135	0.4581	0.4835
Average	0.913267	0.943367	0.9840	Average	0.414583	0.454317	0.483983
Standard deviation	0.001826	0.002501	0.003041	Standard deviation	0.003174	0.002859	0.002997
%RSD	0.199953	0.265107	0.30905	%RSD	0.765565	0.629288	0.619224

Ruggedness:Table 6 clearly indicates that the current method is devoid of analyst effect there within.

Table 6. Ruggedness results of Fluconazole & Ivermectin

Fluconazole			Ivermectin		
Concentration (µg/ml)	**Analyst-1**	**Analyst-2**	**Concentration (µg/ml)**	**Analyst-1**	**Analyst-2**
25	0.9107	0.9715	1	0.4181	0.4814
25	0.9112	0.9713	1	0.4183	0.4815
25	0.9121	0.9797	1	0.4191	0.4818
25	0.9135	0.9717	1	0.4193	0.4816
25	0.9162	0.9778	1	0.4195	0.4819
25	0.9165	0.9711	1	0.4197	0.4811
Average	0.913367	0.97385	Average	0.4190	0.48155
Standard deviation	0.002501	0.003848	Standard deviation	0.000654	0.000288
%RSD	0.273815	0.395131	%RSD	0.156138	0.059827

% Assay (In combination)

Ten tablets were precisely weighed, transported to a clean, dry mortar, and ground into a powder, fine. This powdered tablet was then measured to be 10mg(10.13mg), transferred to a volumetric flask of 10 ml, and the results were recorded. dissolved after adding 5 ml of diluent and sonicating for 15 minutes (1000 µg/ml). Once 1ml of this solution had been transferred to a 10ml flask, using methanol as a diluting agent, it produced 100 µg/ml. 10ml of the flask were filled with 1ml of the a for mentioned solution to create 10 µg/ml.

Vierotd's Method

It is the method in which the sample containing two absorbing species each of which should have some absorbance at λmax of other

Cx= A1ay2 – A2ay1 / ax1ay2 – ax2ay1 Cy = A1ax2 – A2ax1 / ay1ax2 – ay2ax1

Cx=Concentration of Fluconazole
Cy=Concentration of Ivermectin

A1=Absorbance of Fluconazole

A2=Absorbance of Ivermectin

ax1=Absorbance of Fluconazole at 261nm

ax2=Absorbance of Fluconazole at λ max of Ivemectin 245nm

ay1=Absorbance of Ivermectin at 245nm

ay2=Absorbance of Ivermectin at λ max of Fluconazole 261nm

Where as,

A1=0.1145 ax1=0.0239 ay1=0.0602

A2=0.9350 ax2=0.082 ay2=0.09350

By substituting above values, Cx and Cy were found to be 8.8625 and 0.3545 respectively.

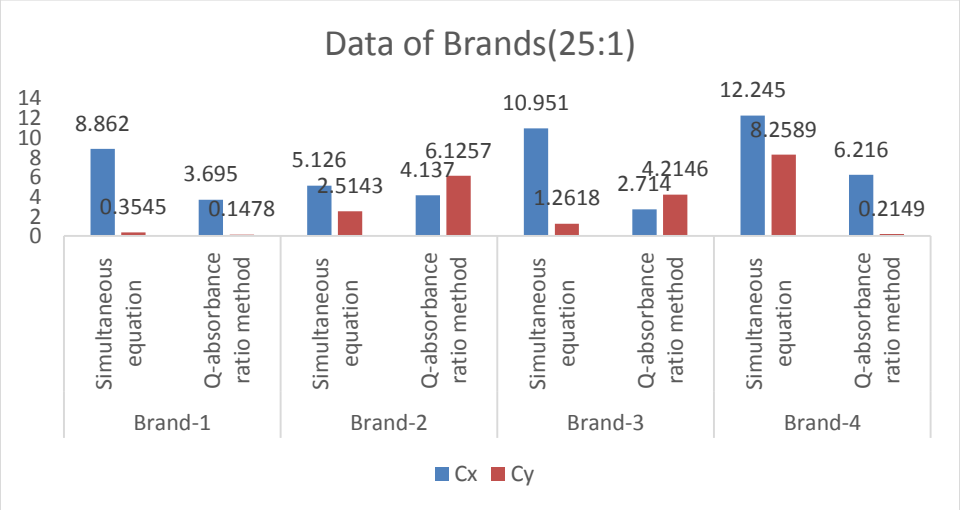

Figure 6. Cx &Cy values of Different brands

Q-Absorbance ratio method

It is the measurement of absorbance at two different wavelengths where one of the wavelengths is λmax of one drug and other wavelength is Iso-bestic point.

Cx = (Qm-Qy / Qx-Qy). A1/ax1

Cy = (Qm-Qx / Qy-Qx). A2/ay1

Qm=A2/A1

Qx=ax2/ax1

Qy=ay2/ay1

A1 & A2 are absorbance of mixture at 245nm & isobestic point.

ax1 & ay1 are absorptivities of Fluconazole and Ivermectin at 245nm

ax2 & ay2 are absorptivities of Fluconazole and Ivermectin at isobestic point.

Where,

A1=0.1145 ax1=0.0239 ay1=0.0602

A2=0.9350 ax2=0.082 ay2=0.09350

Cx and Cy were found to be 3.872 and 0.1478 by substituting the above values. Locally available formulation brands were chosen, and the developed method was used, with the corresponding results shown in Figure 6.

Table7. Various Types of degradation and their condition

Degradation Type	Concentration Reagent	Time
Acid	1N HCL,0.1NHCL	3hrs
Base	1 NaOH,0.1N NaOH	3hrs
Oxidative Hydrolysis	30%H_2O_2	30min,1hr,2hr
Thermal Hydrolysis	45,50,60℃	1hour
UV-degradation	Exposure of drug in UV light.	24hrs
Freezthaw technique	Storing of drug in freezer and bringing back it to room temperature.	24hrs

Graphical representation as shown in Figure 7 involving percentage degraded was observed to be maximum for Ivermectin when subjected to degradation analysis.

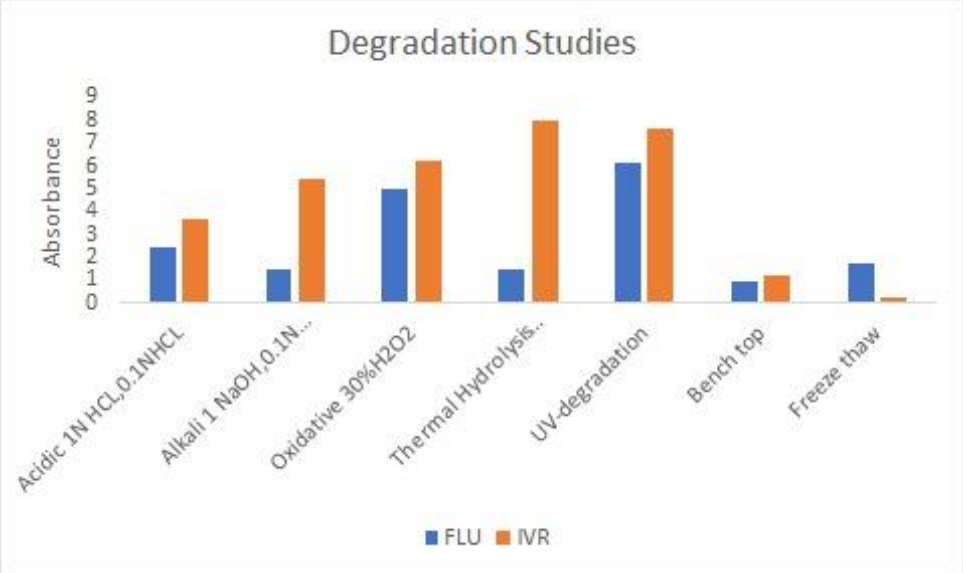

Figure7. Forced degradation results of Fluconazole & Ivermectin

Forced degradation studies

1) **Acid Hydrolysis:** A 1 ml aliquot of solution was added to a 10 ml volumetric flask from a stock solution. Then, it was neutralised with 0.1N NaOH after being combined with 1 ml of 0.1NHCl and allowed to stand for three hours. After being diluted to the proper amount and filtered at 261 and 245 nm for UV spectroscopy, the final concentration of FLU and IVER was 10 µg/ml.

2) **Alkali Hydrolysis:** 1 ml of the stock stock solution was added to a volumetric flask that held 10 ml. After the solutions were allowed to stand for three hours to neutralise the 0.1 N HCl, this was combined with 1 ml of 0.1 N NaOH. After being diluted up to the desired level with a diluent, the final concentration of the FLU and IVER was 10 µg/ml.

3) **Oxidative Hydrolysis:** 10 ml of the volumetric flask were combined with 1 ml of the stock solution and 1 ml of 30% hydrogen peroxide, and the mixture was let to stand for 6 hours. In order to get the appropriate concentration of 10 µg/ml of FLU and IVER, the contents were eventually diluted using diluent.

4) **Thermal Degradation:** 100 mg were weighed and stored for 24 hours at 45–70°C in an oven. The 10 mg sample was weighed and put into a 10 ml volumetric flask. The volume was adjusted to the appropriate level using diluent. 1ml of the aforementioned solution was obtained and transferred into a 10ml volumetric

flask. The volume was then increased to the appropriate amount with diluents. Then, 261nm & 245nm scans of the FLU and IVER solutions were performed.

5) **UV degradation study:** After 24 hours of keeping 100 mg of the sample in a UV chamber set to 200–400 nm, A 10 ml volumetric flask was filled with 10 mg of the sample after it had been weighed. Use diluent to sufficiently amplify the volume. Then transfer 1 ml of the aforementioned solution into a volumetric flask measuring 10 ml. Use diluent to increase the volume. Then, 261nm and 245nm scans of the FLU and IVER solutions were performed.

6) **Bench Top Hydrolysis:** IVER and FLU solutions were scanned at initial, 24 and 48 hours at 261 and 245 nm using 10 ml of volumetric flask with 1 ml of stock solution in it that had been diluted to the appropriate level.

7) **Freezthaw Technique:** During freeze-thaw testing, the product is subjected to freezing temperatures (about -10 °C) for 24 hours, followed by 24 hours of thawing at normal temperature.

4. Summary and Conclusion

The suggested approach is applicable to routine analysis to simultaneously determine the presence of Fluconazole and Ivermectin in API as well as in pharmaceutical dosage forms. It is straightforward, sensitive, accurate, and reproducible. The outcomes of the statistical analysis show how accurate and precise the procedure is. All parameters' relative standard deviations (RSDs) were discovered to be under two, proving the method's validity. The technique can be used for routine analysis of Fluconazole and Ivermectin using Simultaneous estimation and Q-absorption approach. We performed on four different types of brands, all of them has shown the good values of Cx & Cy for the taken ratio of FLU &IVR.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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