DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-UPLC METHOD FOR THE DETERMINATION OF FLUCONAZOLE IN BULK DRUG AND IN PHARMACEUTICAL DOSAGE FORMS

Vamsi Krishna Penmatsa, Basavaiah Kanakapura*, Swamy Nagaraju

Department of Chemistry, University of Mysore, Manasagangotri,

Mysore-570006, Karnataka, India.

*Corresponding Author Email: kanakapurabasavaiah@gmail.com

ABSTRACT

Fluconazole(FLK) is a synthetic triazole derivative antifungal agent that has been shown to be effective against a wide range of systemic and superficial fungal infections. A simple, cost effective, precise, accurate, and stability-indicating isocratic Ultra Performance Liquid Chromatographic (UPLC) method was developed and validated for the determination of FLK in bulk drug and in its tablets. The use of UPLC, with a rapid 5-minute-reversed-phase isocratic separation on a 1.7 µm reversed-phase packing material to provide rapid "high throughput" support for FLK, is demonstrated. The method was developed using Waters Acquity UPLC BEH C18 column (100 mm× 2.1 mm, 1.7 µm) with mobile phase consisting of a mixture of water and acetonitrile (80:20 v/v). The eluted compound was detected at 210 nm with a UV detector. The standard curve of mean peak area versus concentration showed an excellent linearity over a concentration range 0.3–80 µg.mL^{-1} FLK with regression coefficient (r^2) value of 0.9999. The limit of detection (S/N = 3) was 0.1 µg mL^{-1} and the limit of quantification (S/N = 10) was 0.3 µg mL^{-1}. Forced degradation of the bulk sample was conducted in accordance with the ICH guidelines. Acidic, basic, hydrolytic, oxidative, thermal, and photolytic degradations were used to assess the stability indicating power of the method. The drug was found to be stable in acidic, basic, thermal, hydrolytic, and photolytic stress conditions and showed slight degradation in oxidative stress condition.

KEY WORDS

Fluconazole, Pharmaceuticals, Stability indicating, Ultra performance liquid chromatography.

1. INTRODUCTION

Fluconazole (FLK), chemically known as (2-(2, 4,-difluorophenyl)-1, 3-bis (1H1, 2, 4,-triazol-1-yl) propan-2-ol) [1], is a synthetic triazole derivative antifungal agent that has been shown to be effective against a wide range of systemic and superficial fungal infections [2]. The drug is official in United States Pharmacopeia [3], European Pharmacopoeia [4], British Pharmacopoeia [5] which describes non aqueous titration with perchloric acid and the end point being located potentiometrically. The literature survey revealed the availability of many techniques for the assay of FLK in pharmaceuticals. Quantification of FLK has been achieved by high-performance liquid chromatography (HPLC) as alone and as well as in combined formulations [6-19]. Spectrofluorimetry [20], UV-Visbible-spectrophotometry [7, 8, 12, 21-26], high performance thin layer chromatography (HPTLC) [27, 28], thin layer chromatography [29], nuclear magnetic resonance spectroscopy [30] are also reported for the quantification of FLK in formulations. Few Ultra performance liquid chromatographic methods are also reported for the assay of FLK in mixed pharmaceuticals [31, 32].

In recent years, there has been an increasing tendency towards development of stability-indicating assays [33-36]. Chemical and physical degradation of

drugs may result in altered therapeutic efficacy and even toxic effects. Therefore, understanding the factors that change the stability of pharmaceuticals and identifying ways to guarantee their stability are important [37]. Using the approach to stress testing enshrined in International Conference on Harmonisation (ICH) guidelines [38,39]. This approach is being extended to pharmaceuticals to enable accurate and precise quantification of drugs in the presence of their degradation products.

Ultra performance liquid chromatography (UPLC) is a relatively new technique giving new possibilities in liquid chromatography, especially concerning decrease of analysis time and solvent consumption [40]. UPLC system is designed in a special way to withstand high system back-pressure. Special analytical columns UPLC Acquity, UPLC BEH C18 packed with sub 2 μm particles are used in the system. The UPLC system allows shortening analyses time up to nine times compared to the conventional HPLC system, but separation efficiency remains the same or is even improved [41-43].

As efficiency and speed of analyses are of great importance in many applications of liquid chromatography, especially in pharmaceutical, toxicological, and chemical analyses, where it is important to increase throughput and reduce analyses costs, UPLC could play a significant role in the future of liquid chromatography.

Though very few of UPLC methods have earlier been proposed for FLK [31,32], they are suffering from many disadvantages like long runtimes, critical pH adjustments, gradient elution, use of internal standards and lack of stability indicating nature [31,32].

The aim of this work was to develop a rapid, simple, precise, accurate, and validated stability-indicating UPLC method for the determination of FLK in bulk and tablets. This was accomplished with a waters Acquity UPLC system and Acquity BEH column C-18, (100 mm, 2.1 mm, and 1.7 μm). The stability-indicating power of the method was established by comparing the chromatograms obtained under optimized conditions before forced degradation with those after degradation *via* acidic, basic, hydrolytic, oxidative, thermal, and photolytic stress conditions.

EXPERIMENTAL

Chemicals and reagents
Pharmaceutical grade FLK was received from Dr. Reddy's laboratories limited, Hyderabad, India, as gift sample. Two brands of tablets, Nuforce-150 (from Pharma Force Lab, India) and AF-150 (from Ontop Pharmaceuticals pvt. Ltd., India) used in the investigation were purchased from commercial sources in the local market. All the reagents and chemicals used were of analytical reagent grade. HPLC grade acetonitrile was purchased from Merck India, Mumbai, India. Doubly distilled water was used throughout the investigation.

Instrumentation and chromatographic conditions
Chromatographic analysis was carried out on a Waters Acquity UPLC with tunable UV (TUV) detector. Data were collected and processed using Waters Empower software. The separation was performed using an Waters Acquity UPLC BEH C18 column (100 X 2.1 mm, 1.7μm particle size). Isocratic elution process was adopted throughout the analysis with the mobile phase composed of Water and acetonitrile (80:20, v/v). The flow rate was maintained at 0.4 mL min^{-1}. The column effluent was monitored on UV detector set at 210nm. The column temperature was adjusted to 30^{0}C. The injection volume was 4 μL and the run time was 5.0 min. The retention time of the sample was about 1.76 min.

METHODS & GENERAL PROCEDURES

Procedure for preparation of solutions
A stock standard solution of FLK (200 μg mL^{-1}) was prepared by dissolving 20 mg of the pure drug in 100 mL mobile phase. Working solutions were prepared by diluting the stock solution with the mobile phase.

Procedure for preparation of calibration curve
Working solutions equivalent to 0.3 – 80 μg mL^{-1} FLK were prepared by diluting appropriate aliquots of the stock solution. Aliquots of 4 μL of these solutions were injected (triplicate) and eluted with the mobile phase under the stated chromatographic conditions. The average peak area versus the concentration of FLK in μg mL^{-1} was plotted. Alternatively, corresponding regression equation was derived using the mean peak area, concentration data and the

concentration of the unknown was computed from the regression equation.

Procedure for tablets

Twenty tablets were accurately weighed, finely pulverized and mixed using a mortar and pestle. An amount of tablet powder equivalent to 20 mg of FLK was weighed and transferred into a 100 mL volumetric flask, 50 mL of mobile phase was added and was sonicated for 20 min in an ultrasonic bath to complete dissolution of the FLK, the content was then diluted to the mark with the mobile phase, mixed well and filtered using a 0.22 µm nylon membrane filter paper. Aliquots of this solution were successively diluted with the mobile phase and then subjected to analysis as per the general procedure described for the calibration curve.

Procedure for placebo blank analysis

A placebo blank of the composition: Lactose (10 mg) talc (50 mg), starch (50 mg), acacia (50 mg), methyl cellulose (20 mg), sodium citrate (20 mg), magnesium stearate (20 mg) and sodium alginate (10 mg) was made and its solution was prepared as described under 'Procedure for tablets' by taking about 20 mg, and then analyzed using the procedure described earlier.

Procedure for the determination of FLK in synthetic mixture

To ~ 20 mg of the placebo blank of the composition described above, 20 mg of FLK was added and homogenized, transferred to a 100 mL calibrated flask and the solution was prepared as described under "Procedure for tablets", and then subjected to analysis by the procedure described above. This analysis was performed to study the interference by excipients such as lactose, talc, starch, acacia, methyl cellulose, sodium citrate, magnesium stearate and sodium alginate.

Preparation of acid, base and water hydrolysis induced-degradation of sample

For acid, alkaline and water hydrolysis degradation studies to 1.0 mL of 200 µg mL^{-1} stock solution of FLK, 1.0 mL of 5M HCl or 5M NaOH or water were added separately in three 10 mL calibrated flasks. The flasks were kept on a water bath set at 80°C for 3.0 hrs, then cooled to room temperature. Then the solutions were neutralized with equal volume of 5M NaOH or 5M HCl. The content of each flask was made up to the mark with mobile phase. Aliquots of 4 µL of each degraded sample were injected (duplicate) and eluted with the mobile phase under the reported chromatographic conditions.

Preparation of hydrogen peroxide induced-degradation of sample

To 1.0 mL of FLK standard solution (200 µg mL^{-1}) 1.0 mL of 5% hydrogen peroxide was added in 10 mL calibrated flask and kept on a water bath set at 80^{0}C for 3.0 hrs. The flask was cooled to room temperature and made up to the mark with mobile phase. Injected 4 µL of degraded sample in duplicate and eluted with the mobile phase under the reported chromatographic conditions.

Preparation of dry heat degradation and photodegradation samples.

The pure drug was stored in the oven at 105^{0}C for 24 hrs to study dry heat degradation, and exposed to 200 watt hr. m^{-2} UV-radiation and 1.2 million lux hr. of visible radiation for study of photo degradation. Then, solutions equivalent to 20 µg mL^{-1} FLK were prepared in the mobile phase separately from both thermal, photolytic degradation samples and 4 µL of each degraded sample solution was injected in duplicate and eluted with the mobile phase under the optimized chromatographic conditions.

RESULTS AND DISCUSSION

Method development and optimization

Different chromatographic conditions were experimented to achieve better efficiency of the chromatographic system. Parameters such as mobile phase composition, wavelength of detection, column, column temperature, pH of mobile phase, and diluents were optimized. Several proportions of buffer and solvents were evaluated in-order to obtain suitable composition of the mobile phase. Choice of retention time, tailing, theoretical plates, and run time were the major tasks while developing the method. Alternative combinations of gradient and isocratic methods were also performed to obtain a suitable peak. Finally, isocratic method was found suitable for the assay.

Mobile phase selection

Different experiments were carried out to optimize the mobile phase. Several modifications in the mobile

phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the type and ratio of the buffer, organic modifier, buffer pH, the strength of the buffer, and the flow rate. The details of experiments were summarized in **Table 1**. Precise and accurate results with maximum number of theoretical plates and good peak shape were obtained when the mobile phase consisting of water and acetonitrile (80:20, v/v) was used.

Choice of column

Four different columns were used for performance investigations, including Acquity BEH C8 (100 × 2.1 mm i.d, 1.7 μm particle size) column, Acquity BEH Phenyl (100 × 2.1 mm i.d, 2 μm particle size) column, Zorbax Extend C-18 (50 mm x 4.6 mm i.d, 1.8 μm particle size) column, and Waters Aqcquity UPLC BEH C18 (100 mm x 2.1 mm i.d., 1.7 μm particle size) column. The studies revealed that the Waters Aqcquity UPLC BEH C18 column was more suitable since it gave better sensitivity.

Choice of wavelength

Shimadzu pharmaspec UV-1700 UV/Visible spectrophotometer was used for absorbance measurements. A 50 μg mL^{-1} of FLK solution in mobile phase was scanned from 400 to 200 nm against mobile phase as blank and wavelength of the method was optimized as 210 nm.

Choice of other chromatographic conditions

The column oven temperature was studied at higher (40^{0}C) and room (25^{0}C) temperatures and then found that 30^{0}C is the optimum. Flow rate of 0.4 mL min^{-1} was selected with regard to the backpressure and analyses time as well.

Stability study

The degradation study was based on the comparison of the FLK peak area of "stressed FLK samples" with that of the "standard FLK solution". Diluent used for sample preparation (**Figure 1**) and FLK standard solution (**Figure 2**) were injected and eluted with the mobile phase under the optimum chromatographic system. FLK samples treated with acid, base, water, hydrogen peroxide, dry heat and UV-Visible radiation were injected (duplicate) into the chromatographic system and eluted with the mobile phase under the optimum chromatographic conditions (**Figure 3**).

On comparison of FLK stressed sample chromatograms with that of the un-stressed it can conclude that FLK is quite stable under acid, base, water, dry heat and photolytic stress conditions, and unstable under oxidative stress condition.

Analytical parameters validation
Linearity

Stock standard solution of FLK (200 μg mL^{-1}) was appropriately diluted with the mobile phase to obtain solutions in the concentration range 0.3 – 80 μg mL^{-1} FLK. Four microlitre of each solution was injected in triplicate onto the column under the operating chromatographic conditions described above. The least squares method was used to calculate the slope, intercept and the regression coefficient (r^2) of the regression line. The relation between mean peak area Y (n=3) and concentration, X expressed by the equation Y = 18603.80604 X + 2531.61573 (r^2=0.99993), was linear. Related statistical data are presented in **Table 2**.

Limit of quantification (LOQ) and detection (LOD)

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH recommendations [44], below which the calibration graph is non linear and was found to be 0.3 μg mL^{-1}. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected and it was found to be 0.1 μg mL^{-1}.

Selectivity

A systematic study was performed to determine the effect of matrix by analyzing the placebo blank (Figure 4) and synthetic mixture containing FLK (**Figure 5**). Method selectivity was checked by comparing the chromatograms obtained for placebo blank (**Figure 4**), pure FLK solution (**Figure 3**), synthetic mixture and tablet solution (**Figure 5, 6**). An examination of the chromatograms of the above solutions revealed the absence of peaks due to additives present in tablet preparations. The peak area value resulting from 20

µg mL^{-1} FLK in synthetic mixture solution had nearly the same as that obtained for pure FLK solutions of identical concentration. This unequivocally demonstrated the non-interference of the inactive ingredients in the assay of FLK. Further, the slopes of the calibration plots prepared from the synthetic mixture solutions were about the same as those prepared from pure drug solutions.

Precision and Accuracy

Method precision was evaluated from the results of seven independent determinations of FLK at three different concentrations, 10.0, 20.0 and 40.0 µg mL^{-1} FLK on the same day and on five consecutive days. The inter-day and intra-day relative standard deviation (RSD) values for peak area and retention time for the selected concentrations of FLK were less than 2%. The method accuracy, expressed as relative error (%) was determined by calculating the percent deviation found between concentrations of FLK injected and concentrations found from the peak area. This study was performed by taking the same three concentrations of FLK used for precision estimation. The intra-day and inter-day accuracy (expressed as %RE) was less than 2% and the values are compiled in **Table 3**.

Robustness and Ruggedness

To determine the robustness of the method small deliberate changes in the chromatographic conditions like column temperature, flow rate, mobile phase composition and detection wavelength were made, and the results were compared with those of the optimized chromatographic conditions. In each case, the %RSD values were calculated for the obtained peak area and retention time. The results of this study expressed as %RSD are summarized in **Table 4**. At the deliberate varied chromatographic conditions (temperature, detector wavelength, flow rate, and mobile phase composition), the analyte peak area and retention time %RSD remained near to the actual values. The RSD values ranged from 0.12 to 0.50% resumes the robustness of the proposed method. To study the ruggedness of the method, three different columns of same dimensions were used for the analyses. The studies were performed on the same day and on three different days by three different analysts for three different concentrations of FLK (triplicate injections). In each case the %RSD values were calculated for the obtained peak area and retention time. The results of this study expressed as %RSD are summarized in **Table 4**. The analyte peak area and retention time %RSD remained closer to the values under optimized condition.

Application to tablets

The developed and validated method was applied to the assay of FLK in commercial tablets. The results shown in Table 5 are in good agreement with the label claim and with those obtained with the reference method [3]. The reference method involved titration with standard perchloric acid in acetic acid medium, the end point being located potentiometrically. The results showed that the Students's t- and F-values at 95% confidence level did not exceed the tabulated values, which confirmed that there is a good agreement between the results obtained by the proposed methods and the reference method with respect to accuracy and precision. Figure 6 shows a chromatogram indicating good peak due to FLK from the tablet. Therefore, the proposed method can be used for the quality control of the tablets.

Recovery study

To further assess the accuracy and reliability of the method, recovery studies *via* standard addition method was performed. To the pre-analyzed tablet powder, pure FLK was added at three levels and the total was found by the proposed method. Each test was triplicated. When the test was performed on two different brands of tablets, the percent recovery of pure FLK was in the range of 99.3 – 99.9 with standard deviation values of 0.02 – 0.24. The results indicated that the method is very accurate and that common excipients found in tablet preparations did not interfere. The results are complied in **Table 6**.

Table 1:

Solvent A	Solvent B	Observations
Monobasic potassium phosphate (pH-2.5 with 10% H_3PO_4)	Methanol	Broad peak with tailing
Monobasic potassium phosphate (pH-2.5 with 10% H_3PO_4)	Acetonitrile	Peak eluting very early and peak splitting
10 mM ammonium acetate (pH-5.0 with dilute acetic acid)	Methanol	Broad peak & Very less number of theoretical plates
10 mM ammonium acetate (pH-5.0 with dilute acetic acid)	Acetonitrile	Peak eluting early and peak splitting
Water	Methanol	Broad peak & Very less number of theoretical plates
Water	Acetonitrile	Good peak shape with theoretical plates more than 9000

Table 2: Sensitivity and regression parameters

Parameter	Value
Linearity range, $\mu g\ mL^{-1}$	0.3 - 80
Regression ($Y^* = a + bX$)	
Slope (b)	18603.79
Intercept (a)	2532.59
Regression co-efficient (r^2)	0.9999
Limit of detection (LOD, $\mu g\ mL^{-1}$)	0.1
Limit of quantification (LOQ, $\mu g\ mL^{-1}$)	0.3

*$Y = a+bX$, where Y is the area and X concentration in $\mu g\ mL^{-1}$.

Table 3: Intra-day and inter-day accuracy and precision.

FLK Injected, $\mu g\ mL^{-1}$	Intra-day accuracy and precision				Inter-day accuracy and precision			
	FLK found[a]	%RE	%RSD[b]	%RSD[c]	FLK found[a]	%RE	%RSD[b]	%RSD[c]
5	4.92	-1.6	1.42	0.029	4.91	-1.8	1.34	0.044
20	20.08	0.40	0.35	0.028	20.04	0.20	0.87	0.033
80	80.14	0.18	0.42	0.045	80.09	0.11	0.11	0.044

a-Mean value of seven determinations

b-Based on peak area.

c-Based on retention time

Table 4: Results of Robustness and Ruggedness study (FLK concentration, 20 µg mL^{-1}, n = 3)

Condition	Modification	Mean peak area ±S D			%RSD	Mean Rt ± SD			%RSD
Optimized condition	----	374896	+	481.0	0.13	1.766	+	0.001	0.03
Column Temperature (°C)	29	373755	+	777.7	0.21	1.773	+	0.001	0.03
	31	376080	+	501.3	0.13	1.753	+	0.001	0.03
Detector Wavelength (nm)	209	380690	+	615.6	0.16	1.764	+	0.001	0.03
	211	368413	+	1367.7	0.37	1.766	+	0.001	0.04
Mobile phase flow rate (mL min^{-1})	0.39	371915	+	1863.7	0.50	1.853	+	0.001	0.03
	0.41	374731	+	1165.3	0.31	1.702	+	0.001	0.03
Mobile phase composition (v/v)	Water : ACN (78:22)	371296	+	1117.6	0.30	1.712	+	0.001	0.03
	Water : ACN (82:18)	375994	+	794.9	0.21	1.862	+	0.001	0.03
Different Analyst	Analyst-1	374112	+	899.1	0.24	1.782	+	0.001	0.03
	Analyst-2	382305	+	1596.5	0.42	1.811	+	0.001	0.03
	Analyst-3	376511	+	1154.7	0.31	1.756	+	0.001	0.03
Different Column	Column-1	380556	+	599.2	0.16	1.712	+	0.001	0.03
	Column-2	377152	+	816.9	0.22	1.766	+	0.001	0.03
	Column-3	376502	+	215.7	0.06	1.782	+	0.001	0.03
Different Day	Day-1	371154	+	562.0	0.15	1.853	+	0.001	0.05
	Day-2	378590	+	444.4	0.12	1.782	+	0.001	0.03
	Day-3	382100	+	1744.2	0.46	1.782	+	0.001	0.05

Table 5: Results of determination of FLK in tablets and statistical comparison with the reference method.

Tablet brand name	Nominal amount, mg	Found* (Percent of label claim ± SD)	
		Reference method	Proposed method
AF-150	150	99.5 ± 0.93	100.3 ± 0.47 t = 1.73 F = 3.89
NUFORCE-150	150	98.7 ± 1.26	99.5 ± 0.99 T = 1.18 F = 1.62

*Mean value of five determinations

Tabulated t-value at the 95% confidence level is 2.77

Tabulated F-value at the 95% confidence level is 6.39

Table 6: Results of recovery study by standard addition method

Tablet	FLK in tablet, µg mL^{-1}	Pure FLK added, µg mL^{-1}	Total found, µg mL^{-1}	Pure FLK recovered*, Percent ± SD		
	10.12	10	20.04	99.6	±	0.23
AF-150	10.12	20	30.06	99.8	±	0.25
	10.12	40	49.97	99.7	±	0.02
	10.05	10	19.91	99.3	±	0.15
NUFORCE-150	10.05	20	30.03	99.9	±	0.12
	10.05	40	50.02	99.9	±	0.18

*Mean value of three determinations
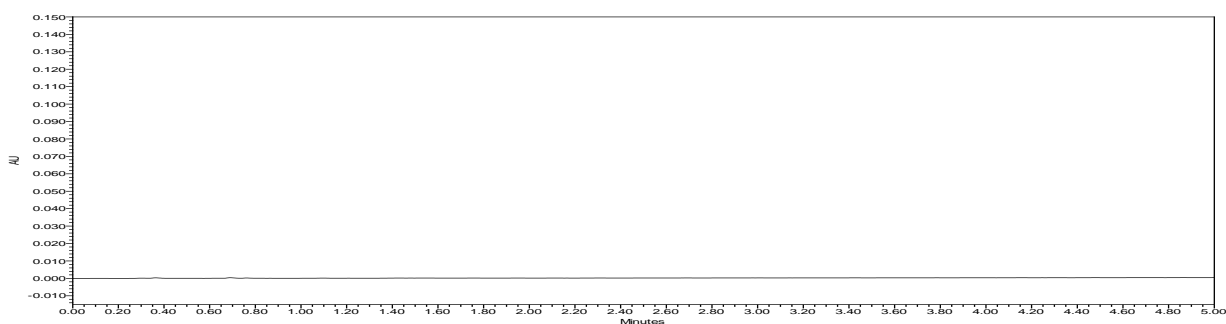
Figure (1): Blank chromatogram
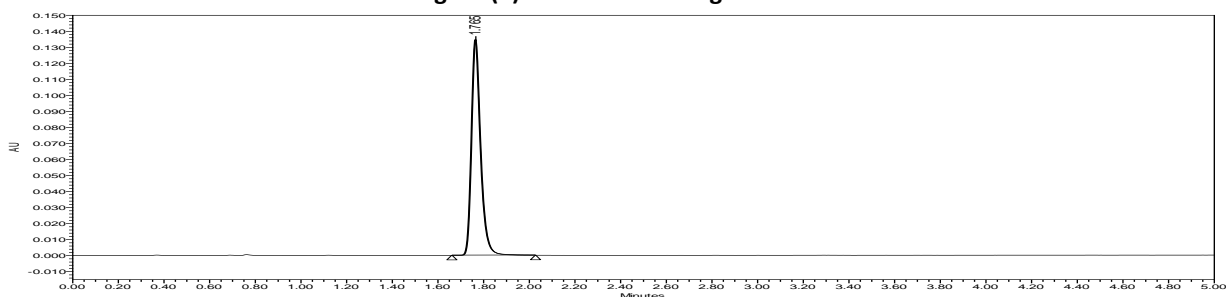
Figure (2): Fluconazole standard chromatogram (20 µg mL^{-1})
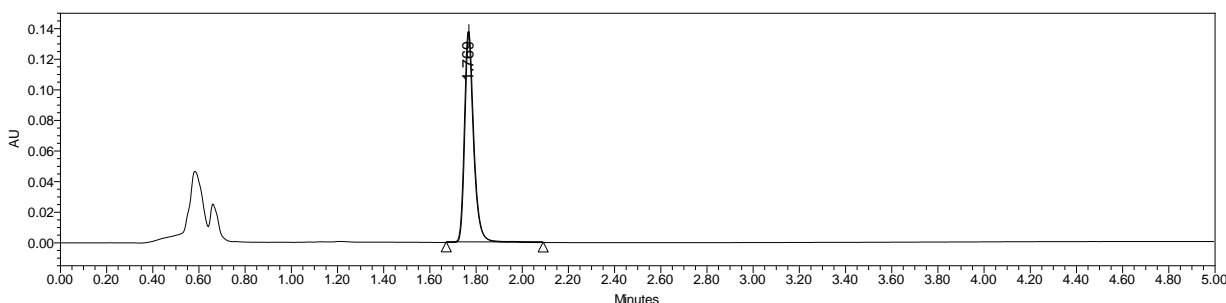
Figure (3a) : FLK acid degradation sample chromatogram
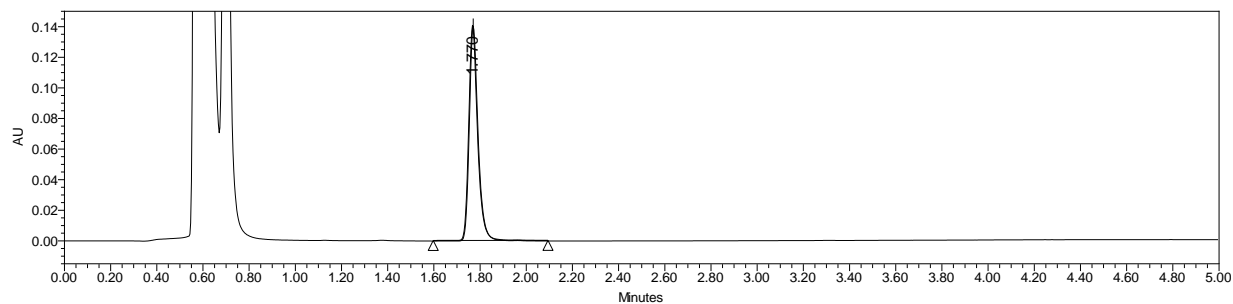
Figure (3b): FLK base degradation sample chromatogram
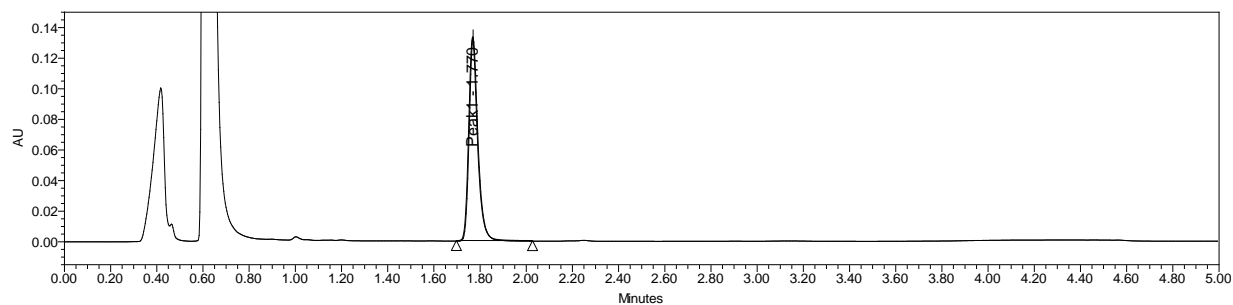
Figure (3c): FLK peroxide degradation sample chromatogram
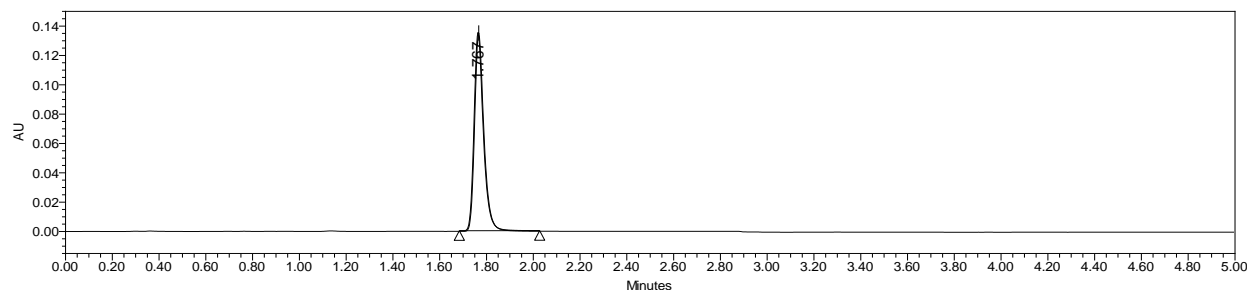
Figure (3d): FLK thermal degradation sample chromatogram
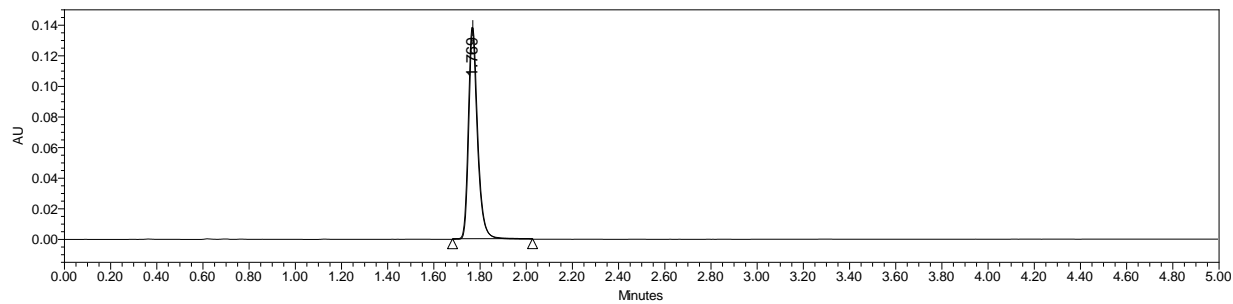

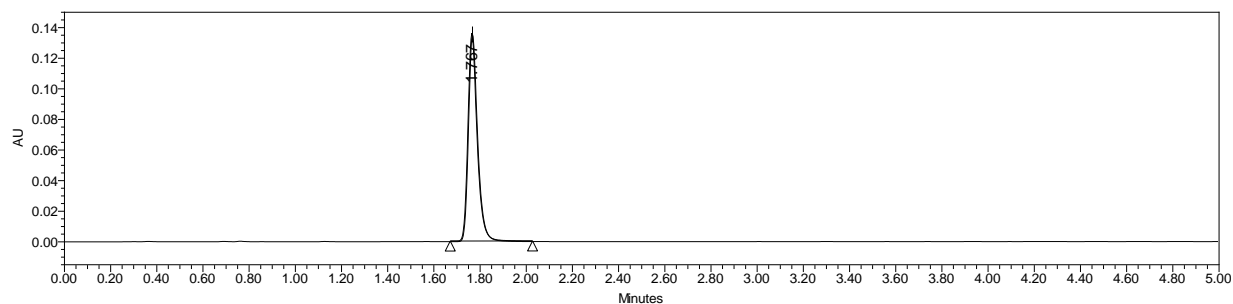**Figure (3e): FLK water hydrolysis degradation sample chromatogram**

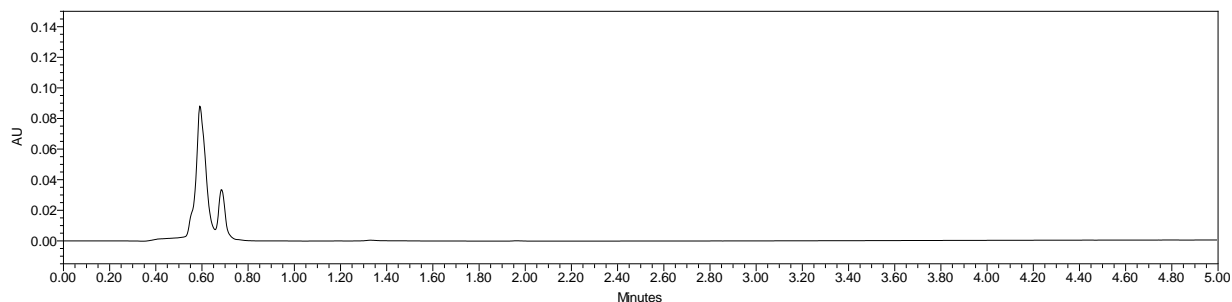**Figure (3f): FLK photolytic degradation sample chromatogram**
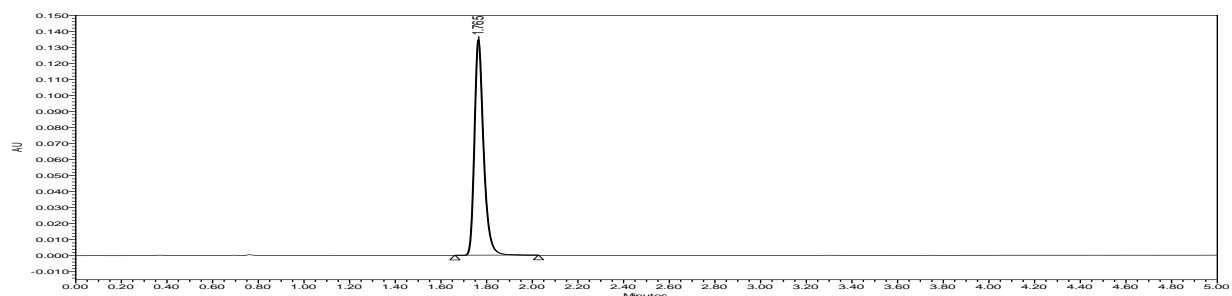
Figure (4) : placebo blank chromatogram

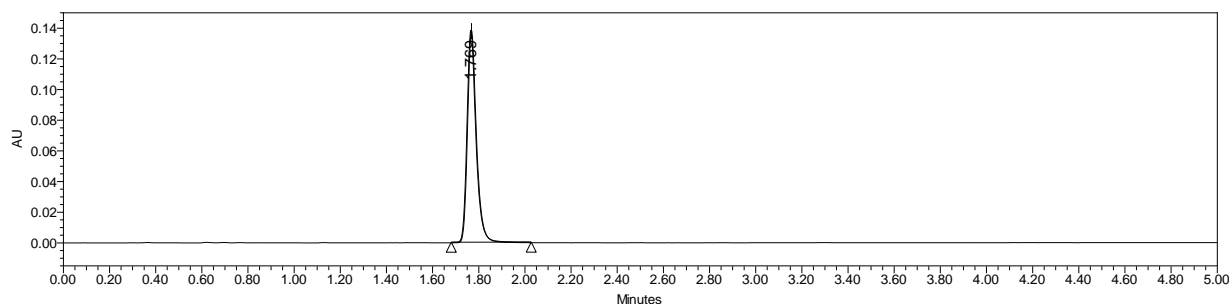**Figure (5): synthetic mixture chromatogram**

Figure (6): Tablet chromatogram

CONCLUSION

In this work, a stability-indicating reverse phase isocratic UPLC method was developed and validated for the determination of FLK in bulk drug and in tablets. The retention time obtained for FLK (1.7 min) enables rapid determination of the drug which is important in routine analysis. The method exhibited an excellent performance in terms of sensitivity, linearity and speed. The method is stability indicating and can be used for routine analysis of production samples and can be used for the assay of fluconazole either in pure drug or pharmaceutical formulations. Degradation study of the method reveals that the product is unstable in peroxide medium. The proposed method is superior over other reported methods in terms of stability indicating nature which is most important requirement for an assay method, less volumes solvents were used in mobile phase, lower flow rate and with less run time. No corrosive solvents or ion pairing reagents were used which lead to longer column equilibration time and reduces the column life. No internal standards were used.

ACKNOWLEDGEMENT

REFERENCES

1. *The Merck Index*, 14th Edition, Merck & co., Inc., Whitehouse Station, N.J.:; Monograph No. 0004122, (2006).

2. Martindale, *The extra Pharmacopoeia*, ed, Reynolds, J.E.F. 31ST Edition, Royal Pharmaceutical Soc, London: 404-406, (1996).

3. *United States Pharmacopeia*, USP35, National formulary-32, Rockville, USP Convention, Fluconazole: 3204-3205, (2012).

4. *European Pharmacopoeia*, EDQM, Council of Europe, Strasbourg, France, Edition 7.6.: 4936-4937, (2012).

5. *British Pharmacopoeia*, Her Majesty's, Stationery office, London; 1&2. Monograph no. 2287, (2009).

6. Abdel-Aleem, AA, Lotfy HM, Monir HH. Stability-indicating high performance liquid chromatographic determination of fluconazole in the presence of its oxidative degradate-kinetic and stress study. *International Research Journal of Pharmaceuticals*, *2(1)*:6-12, (2012).

7. Correa JCR, Vianna-Soares CD, Salgado HRN. Development and validation of dissolution test for fluconazole capsules by HPLC and derivative UV spectrophotometry. *Chromatography Research Internationa, Article i.d*. 610427:1-8, (2012).

8. Correa JCR, Reichman C, Salgado HRN, Vianna-Soares Cristina Duarte. Performance characteristics of high performance liquid chromatography, first order derivative UV spectrophotometry and bioassay for fluconazole determination in capsules. *Quimica Nova, 35(3):*530-534, (2012).

9. Correa JCR, Reichman C, Vianna-Soares CD, Salgado HRN. Stability study of fluconazole applying validated bioassay and stability-indicating LC methods. *Journal of Analytical & Bioanalytical Techniques*, *2(5)*:126, (2011).

10. Queiroz KM, Silva MLM, Prado N, Duque, Lima PMA, Diniz RDL, Cesar IC. et al.. Comparison of microbiological assay and HPLC-UV for determination of fluconazole in capsules. *Brazilian Journal of Pharmaceutical Sciences*, *45(4)*:693-700, (2009).

11. Al-Rimawi F. Development and validation of analytical method for Fluconazole and Fluconazole related compounds (A, B, and C) in capsule formulations by HPLC with UV detection. *Jordan Journal of Chemistry*, *4(4)*:357-365, (2009).

12. Sadasivudu P, Shastri N, Sadanandam M. Development and validation of RP-HPLC and UV methods of analysis for fluconazole in pharmaceutical solid dosage forms. *International Journal of ChemTech Research, 1(4)*:1131-1136, (2009).

13. Hurtado FK, Souza MJ, de Melo J, Rolim CMB. Microbiological assay and HPLC method for the determination of fluconazole in pharmaceutical injectable formulations. *Latin American Journal of Pharmacy*, *27(2):*224-228, (2008).

14. Wallace JE, Harris SC, Gallegos J, Foulds G, Chen TJH, Rinaldi MG. Assay of fluconazole by high-performance liquid chromatography with a mixed-phase column. *Antimicrobial Agents and Chemotherapy*, *36(3):*603-606, (1992).

15. [15] Elezovic A, Elezovic A, Hadzovic S. Simple, inexperience and ecologically friendly derivative spectrophotometric fluconazole assay from nail lacquer formulations. *American Journal of Analytical Chemistry*, *2(2)*:109-115, (2011).

16. Harod SS, Manocha N, Hingole A, Dubey PK. Development and validation of analytical method for Fluconazole and Ivermectin in tablet formulation by using RP-HPLC. *International Research Journal of Pharmacy*, *3(8)*:257-261, (2012).

17. Bodepudi C, Bantu S, Kalyan ORM, Shanmugasundaram P, Vijey AM. Novel reverse phase HPLC method development and validation of fluconazole and tinidazole in a combined tablet dosage form. *International Journal of ChemTech Research*, *3(3)*:1309-1317, (2011).

18. Meshram DB, Bagade SB, Tajne MR. Simple HPLC method for simultaneous estimation of fluconazole and tinidazole in combined dose tablet. *Journal of Chromatographic Science*, *47(10)*:885-888, (2009).

19. Abdel-Moety EM, Khattab FI, Kelani KM, Abou Al-Alamein AM. Chromatographic determination of clotrimazole, ketoconazole, and fluconazole in pharmaceutical formulations. *Farmaco*, *57(11)*:931-938, (2002).

20. El-Bayoumi A, El-Shanawany AA, El-Sadek ME, El-Sattar AA. Synchronous spectrofluorometric determination of famotidine, fluconazole and ketoconazole in bulk powder and in pharmaceutical dosage forms. *Spectroscopy Letters*, *30(1)*:24-46, (1997).

21. Alizadeh N, Rezakhani Z. Extractive spectrophotometric determination of ketoconazole, clotrimazole and fluconazole by ion-pair complex formation with bromothymol blue and picric acid. *Journal of the Chilean Chemical Society*, *57(2)*:1104-1108, (2012).

22. Singh A, Sharma PK, Majumdar DK. Development and validation of different UV-spectrophotometric methods for the estimation of fluconazole in bulk and

in solid dosage form. *Indian Journal of Chemical Technology*, *18(5)*:357-362, (2011).

23. Gondalia RP, Patel DP, Savaliya PJ. Development and validation of UV spectrophotometric method for estimation of fluconazole in soft gelatin capsule. *International Journal of Pharmacy and Technology*, *2(4)*:938-944, (2010).

24. Jalali F, Rajabi MJ. Extractive spectrophotometric determination of fluconazole by ion-pair complex formation with bromocresol green. *Chinese Journal of Chemistry*, *25(9)*:1300-1303, (2007).

25. Aboul-Enein HY, Goger NG, Turkalp A. Quantitative determination of fluconazole in syrups by first order derivative spectrophotometry. *Analytical Letters*, *35(7)*:1193-1204, (2002).

26. Goger NG, Aboul-Enein HY. Quantitative determination of fluconazole in capsules and IV solutions by UV spectrophotometric methods. *Analytical Letters*, *34(12)*:2089-2098, (2001).

27. Shewiyo DH, Kaale E, Risha PG, Sillo HB, Dejaegher B, Smeyers-Verbeke J. et al.. Development and validation of a normal-phase HPTLC-densitometric method for the quantitative analysis of fluconazole in tablets. *Journal of Planar Chromatography-Modern TLC*, *24(6)*:529-533, (2011).

28. Ramesh B, Narayana PS, Reddy AS, Devi PS. Spectrodensitometric evaluation and determination of fluconazole and its impurities in pharmaceutical formulations by high performance thin layer chromatography. *Journal of Pharmacy Research*, *4(5)*:1401-1404, (2011).

29. Meshram DB, Bagade SB, Tajne MR. A simple TLC method for analysis of fluconazole in pharmaceutical dosage forms, *Journal of Planar Chromatography-Modern TLC*, *21(3)*:191-195, (2008).

30. Senyuva H, Ozden T, Aksahin I. Quantitative determination of fluconazole in solid dosage forms by 1H-NMR spectroscopy. *Journal of Faculty of Pharmacy of Gazi University*, *18(2)*:107-113, (2001).

31. Yanamandra R, Chaudhary A, Bandaru SR, Sastry CSP, Patro Balaram, Murthy YLN. et al.. Development of a RP-UPLC method for the simultaneous analysis of secnidazole, fluconazole, and azithromycin: application in pharmaceuticals and human serum. *International Journal of PharmTech Research, 3(2):*1198-1207, (2011).

32. Yanamandra R, Chaudhary A, Srinivasa RB, Patro B, Murthy YLN, Ramaiah PA, Sastry CSP. UPLC method for simultaneous separation and estimation of secnidazole, fluconazole and azithromycin in pharmaceutical dosage forms. *E-Journal of Chemistry*, *7(1)*:363-371, (2010).

33. Singh S, Singh B, Bahuguna R, Wadhwa L, Saxena R. Stress degradation studies on ezetimibe and development of a validated stability-indicating HPLC assay. *Journal of Pharmaceutical and Biomedical Analysis*, *41(3)*:1037–1040, (2006).

34. Mohammadi A, Haririan I, Rezanour N, Ghiasi L, Walker RB. A stability-indicating high performance liquid chromatographic assay for the determination of orlistat in capsules. *Journal of Chromatography A*, *1116:*153–157, (2006).

35. Ivana I, Ljiljana Z, Mira Z. A stability indicating assay method for cefuroxime axetil and its application to analysis of tablets exposed to accelerated stability test conditions. *Journal of Chromatography A*, *1119:*209–215, (2006).

36. Vinay KB, Revanasiddappa HD, Xavier CM, Ramesh PJ, Raghu MS. A stability indicating uplc method for the determination of tramadol hydrochloride: application to pharmaceutical analysis. *Chromatography Research International, Article ID 870951*:1-9, (2012).

37. Adams AIH, Gosmann G, Schneider PH, Bergold AM. LC stability studies of voriconazole and structural elucidation of its major degradation product. *Chromatographia*, *69*:115-122, (2009).

38. *International Conference on Harmonization Guideline on Stability testing of new drug substances and products. Q1A (R2)*, (2006).

39. *International Conference on Harmonization Guideline on Photo stability testing of new drug substances and products*. Q1B, (1996).

40. Nov´akov´a L, Matysov´a L, Solich P. Advantages of application of UPLC in pharmaceutical analysis. *Talanta*, *68*:908–918, (2006).

41. Michael ES. UPLC: An Introduction and review. *Journal of Liquid Chromatography & Related Technologies, 28:*1253-1263, (2005).

42. Michael ES. Ultra Performance Liquid chromatography (UPLC): An Introduction, Separation science redefined. *www.chromatographyonline.com,* (2005).

43. Michael ES, Brian JM. Ultra performance liquid chromatography: tomorrow's HPLC technology today. *LPI Journals Holdings.* (2004).

44. *International Conference on Harmonization Guideline on Analytical method validation* Q2 (R1), (2006).

*Corresponding Author:

Dr.K.BASAVAIAH

M.Sc., Ph.D.

Professor in Chemistry
Department of P.G.Studies and Research in Chemistry
University of Mysore
Manasagangothri, Mysore –570 006, INDIA