ACTA SCIENTIFIC PHARMACEUTICAL SCIENCES (ISSN: 2581-5423)

Volume 3 Issue 5 May 2019

Research Article

New Stability Indicating RP-UFLC Method for the Determination of Flucytosine – An Anti Fungal Agent

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Received: April 01, 2019; **Published:** April 12, 2019

Abstract

A new RP-UFLC method was developed for the determination of Flucytosine in its capsules and validated using Shimadzu Model CBM-20A/20 Alite UFLC system (Shimadzu Co., Kyoto, Japan) equipped with SPD M20A prominence photodiode array detector on C8 (2) 100A (Luna) column (250 mm × 4.60 mm i.d. 5µm particle size) maintained at room temperature. Mobile phase consisting of tetra butyl ammonium hydrogen sulphate and acetonitrile (50:50 v/v) was selected with a with flow rate of 1 mL/min (UV detection at 215nm) for the determination of Flucytosine. System suitable parameters are within the acceptable criteria. Flucytosine has shown linearity over the concentration range 0.1–120 µg/mL with linear regression equation y = 107100x- 5776.2 (r2 = 0.9999). The LOQ was found to be 0.5419 µg/mL and the LOD was found to be 0.1786 µg/mL. Flucytosine was subjected to forced degradation and the method was validated as per ICH guidelines.

Keywords: Flucytosine; RP-UFLC; Stability indicating; Validation; ICH Guidelines

Introduction

Flucytosine (FCT) is an antifungal agent used for the treatment of systemic mycotic infections [1]. Chemically it is Flucytosine (4-amino-5-fluoro-2(lH-pyrimidone) [2] and it is a fluorinated analogue of cytosine. FCT (Figure 1) was synthesized in 1957, as a potential anti-tumour agent [2] but it was not sufficiently effective against tumours. Four years later, 5-FC proved to be active in experimental candidosis and cryptococcosis in mice and in 1968, it was used to treat human candidosis and cryptococcosis. In addition to its activity against *Candida* spp. and *Cryptococcus neoformans*, 5-FC is active against fungi causing chromoblastomycosis. FCT itself has no antifungal activity but its antimycotic activity results from the rapid conversion of FCT into 5-Fluoro uracil within susceptible fungal cells. FCT is taken up by these cells by the enzyme cytosine permease, which is also the transport system for adenine, hypoxanthine and cytosine. Monotherapy with FCT is now only used in some cases of chromoblastomycosis and in uncomplicated lower urinary tract candidosis and vaginal candidosis and in all other cases FCT is used together with other agents, usually amphotericin B, for the treatment of systemic mycoses [3].

Flucytosine was determined in biological fluids by HPLC methods [4-6], microbiological assay [7], fluoremetric methods [8] and also in presence of other drugs such as amphotericin B [9], furosemide [10] and fluorouracil in blood plasma [11].There are only two stability indicating methods in the literature in which Mohabbat Ullah., *et al*. studied its impurities in its injectable formulations [12] and Milind Ubale., *et al*. [13] studied by using water : methanol in which the calibration was not so satisfactory. In the present study the authors have selected the work to develop a robust and economic stability indicating method for the quantification of Flucytosine in capsules. The method was validated as per ICH guidelines [14,15].

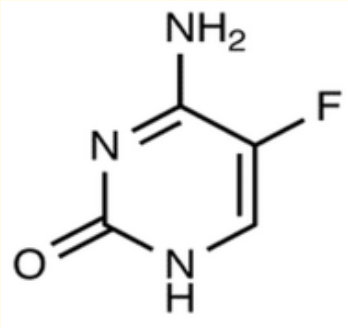

Figure 1: Chemical structure of Flucytosine.

Materials and Methods

Instrumentation

Chromatographic separation was achieved by Shimadzu Model CBM-20A/20 Alite UFLC system (Shimadzu Co., Kyoto, Japan) equipped with SPD M20A prominence photodiode array detector on C8 (2) 100A (Luna) column (250 mm × 4.60 mm i.d. 5µm particle size) maintained at room temperature.

Preparation of mobile phase

3.4 grams of TBHS was transferred to a 1000mL volumetric flask and volume was made up with HPLC grade water. The resulting solution was sonicated for half an hour and filtered through 0.45µm membrane filter prior to injection.

Preparation of Flucytosine drug solution

Accurately weighed 25 mg of Flucytosine was taken in a 25mL volumetric flask and volume is made up to the mark with HPLC grade acetonitrile (1000 µg/mL), and dilutions were made with mobile phase and filtered through 0.45 µm membrane filter prior to injection.

Method validation

Linearity

A series of solutions (0.1–120 µg/mL) FCT stock solution with mobile phase and 20 µL of each of these solutions were injected in to the HPLC system. The mean peak area of FCT were calculated from the chromatograms and a calibration curve was drawn by taking the concentration of the FCT solutions on the x-axis and the corresponding mean peak area values on the y-axis.

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied using three different concentrations of Flucytosine on the same day and on three consecutive days respectively and the % RSD was calculated. The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of FLU in the drug product and the percentage recovery was calculated. The robustness of the method was assessed by exposing the drug solution to different analytical conditions purposely changing from the original optimized conditions. The effects so obtained were summarized to calculate the % RSD and has to be less than 2.0% specifying that the proposed method was robust.

Forced degradation studies

Forced degradation studies were performed to determine the ability of the drug to withstand its properties in the applied stress conditions. Flucytosine was exposed to different stress conditions such as acidic hydrolysis, basic hydrolysis, oxidation, thermal treatment.

Acidic degradation was performed by treating the drug solution with 1mL of 0.1N HCl, heated at 80 ºC for about 30 minutes on a water bath. The stressed sample is then cooled neutralized with 1mL 0.1N sodium hydroxide solution and the solution was made up to volume to the required concentration with the mobile phase. 20 µl of the solution was injected in to the UFLC system.

Alkaline degradation, was performed by treating the drug solution with 1mL 0.1 N NaOH heated at 80ºC for 30 minutes on a water bath. The solution is then cooled and neutralized with 1mL 0.1N hydrochloric acid and diluted with mobile phase. 20 µl of the solution was injected in to the UFLC system.

Oxidation degradation was performed by treating the drug solution with 1ml of 30% v/v H_2O_2 heated at 80ºC for 30 minutes on a water bath. The solution is then cooled and diluted with mobile phase. 20 µl of the solution was injected in to the UFLC system.

Thermal degradation was performed by heating the drug solution at 80ºC for 30 minutes on a water bath. The solution is

then cooled and diluted with mobile phase. 20 µl of the solution was injected in to the UFLC system.

Assay of Flucytosine capsules

Twenty capsules were procured and the contents were powdered and powder equivalent to 25 mg Flucytosine was extracted using the mobile phase in a 25 ml volumetric flask. The solution was sonicated for half an hour and filtered through 0.45 mm membrane filter and 20 µL of this solution was injected in to the UFLC system and the peak area was noted at its retention time from the resultant chromatogram.

Results and Discussion

A new reverse phase ultrafast acting stability indicating method was developed for the quantification of Flucytosine in capsules. The previously reported liquid chromatographic methods were compared with the present method in Table 1. A C8 Luna column (250 mm × 4.60 mm i.d. 5µm particle size) was selected with mobile phase composition TBHS: acetonitrile (50:50 v/v) and flow rate 1 mL/min (UV detection at 215nm) for the determination of Flucytosine by which a sharp peak was observed at 2.875 min (Run time 10 min). The optimized chromatographic conditions were shown in Table 2.

Column	Method/Mobile phase (v/v)	λ_{max}	Comment	Reference
C18 (250 x 4.6 mm, 5µ)	HPLC Water and Methanol (95:5)	260	10-150	12
C18 (250 x 4.6 mm, 5µ)	HPLC (Sodium dihydrogen phosphate: 1-octane Sulfonic acid) Buffer: Methanol: Acetonitrile (50:20:30)	264	22-80	13
C8 (250 x 4.6 mm, 5µ) 100A (Luna)	UFLC TBHS: Acetonitrile (50: 50)	215	0.1-120	Present method

Table 1: Review of previously published methods with the present method.

Parameter	Optimized chromatographic conditions
Mobile Phase	TBHS and Acetonitrile (50: 50 v/v)
Stationary Phase	C8 (2) 100A (Luna) column (250 mm × 4.60 mm i.d. 5µm particle size)
Flow Rate	1 mL/min
Detection wavelength	215 nm
Column temp.	(25°± 2°C)
Injection Volume	20 µL
Detector	SPD M20A prominence photodiode array detector
Elution	Isocratic mode
Total Run Time	10 mins
Retention time	2.857 mins

Table 2: Optimized chromatographic conditions.

Linearity

Flucytosine has shown linearity over the concentration range 0.1–120 µg/mL (Table 3) (% RSD 0.005-0.404) with linear regression equation y = 107100x- 5776.2 (r^2 = 0.9999) (Figure 2). The LOQ was found to be 0.5419 µg/mL and the LOD was found to be 0.1786 µg/mL. The system suitability parameters for the Flucytosine has shown that the tailing factor was less than 2 (or <1.5-2.0) and the theoretical plates were more than 2000.

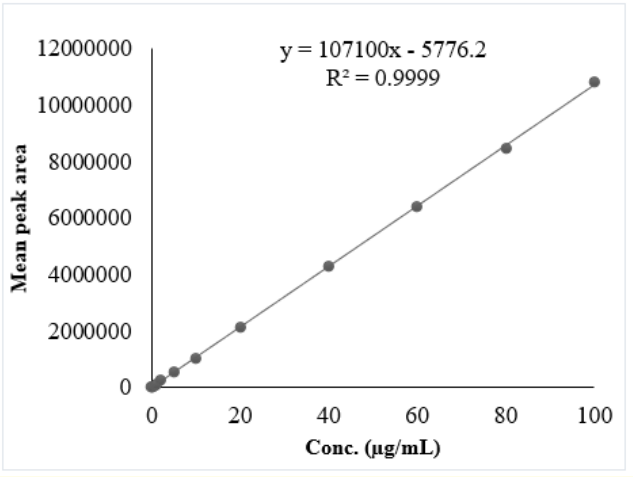

Figure 2: Calibration curve of Flucytosine.

Conc. (µg/mL)	*Mean peak area	% RSD
0.1	11083	0.033
0.2	22167	0.020
0.5	55417	0.033
1	110835	0.030
2	221670	0.020
5	554175	0.073
10	1061062	0.404
20	2122124	0.024
40	4244248	0.105
60	6366372	0.061
80	8488496	0.005
100	10810620	0.038
120	13134684	0.074

*Mean of three replicates

Table 3: Linearity of Flucytosine.

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied at three different concentration levels of Flucytosine on the same day and on three consecutive days respectively and the % RSD was found to be 0.136-0.429 (Intraday) (Table 4) and 0.084-0.533 (Inter day) (Table 5) respectively (<2.0) demonstrating that the method is precise. The accuracy of the method was proved by the standard addition method and the recovery values were 0.19-0.63 (<2.0) with a recovery of 98.91-101.50% (Table 6). The robustness of the assay method was established by introducing small changes in the chromatogaphic conditions which include detection wavelength (213 and 217 nm), percentage of acetonitrile in the mobile phase (48 and 52%) and flow rate (± 0.1 ml/min). Robustness of the method was studied using 10 µg/mL of Flucytosine (Table 7) and the % RSD was found to be 0.70-1.55 (<2.0).

Conc. (µg/mL)	*Mean peak area	Statistical Analysis
		*Mean ± SD (% RSD)
10	1061126	1066740 ± 4583.812 (0.429)
10	1072354	
10	1051024	
20	2123214	2129170 ± 4863.054 (0.228)
20	2135126	
20	2143601	
30	3183186	3177884 ± 4329.473 (0.136)
30	3172581	
30	3163410	

*Mean of three replicates

Table 4: Intraday precision study of Flucytosine.

Conc. (µg/mL)	*Mean peak area			*Mean ± SD (% RSD)
	Day 1	Day 2	Day 3	
10	1061163	1075128	1050132	1068146 ± 5701.187 (0.533)
20	2123214	2122124	2123120	2129468 ± 5179.854 (0.243)
30	3183186	3184816	3183861	3181533 ± 2680.967 (0.084)

*Mean of three replicates

Table 5: Inter day precision study of Flucytosine.

Spiked Conc. (µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Conc. obtained (µg/mL) ± SD (%RSD)	% Recovery
5 (50%)	10	15	14.96 ± 0.005 (0.33)	99.61
	10	15		100.2
	10	15		98.91
10 (100%)	10	20	20.15 ± 0.04 (0.19)	101.5
	10	20		99.92
	10	20		101.1
15 (150%)	10	25	25.1 ± 0.16 (0.63)	101.2
	10	25		99.91
	10	25		101.2

*Mean of three replicates

Table 6: Accuracy study of Flucytosine.

Parameter	Condition	*Mean peak area	*Mean peak area ±SD (% RSD)
Flow rate (± 0.1 ml/min)	0.9	1102356	1081709 ± 16858.2 (1.55)
	1.0	1061062	
	1.1	1015320	
Detection wavelength (± 2 nm)	213	1047469	1038524 ± 7303.5 (0.70)
	215	1029579	
	217	1058423	
Mobile phase composition TBHS: ACN (± 2 %, v/v)	52:48	1056241	1042910 ± 10884.7 (1.04)
	50:50	1029579	
	48:52	1063215	

*Mean of three replicates

Table 7: Robustness study of Flucytosine.

Forced degradation studies

Flucytosine was eluted at 2.875 min. Most of the drug was degraded in alkaline hydrolysis (14.26%) and during oxidation an extra peak was observed at 4.196 min along with the drug peak at 2.847min (drug degradation 2.02%). In thermal degradation the drug peak was eluted at 2.828 min and no degradants were reported. It is confirmed that the drug is sensitive towards alkaline conditions and it was also observed that the drug peak was well separated indicating that the method is selective and specific. The system suitability parameters were well in the acceptance criteria (Table 8,9). The individual chromatograms observed during the forced degradation studies were shown in Figure 3 and the corresponding 3D chromatograms were shown in Figure 4. The peak purity plots were shown in Figure 5 and the overlay chromatograms were shown in Figure 6A.

Stress condition	Rt (min)	% Recovery*	% Drug degradation	Theoretical Plates (>2000)	Tailing factor (<1.5)
Standard drug	2.857	100	------	4823	1.211
Acidic degradation 0.1N HCl/ 80°C/ 30 min	2.829	95.05	4.94	6291	1.237
Alkaline degradation 0.1N NaOH/ 80°C/ 30 min	2.804	85.73	14.26	5268	1.322
Oxidative degradation 30% H_2O_2/ 80°C/ 30 min	2.847 4.196	97.97	2.02	5855	1.256
Thermal degradation Water/80°C/30 min	2.828	99.65	0.34	5549	1.229

*Mean of three replicates

Table 8: Stress degradation studies of Flucytosine.

Brand	Label claim (mg)	Observed amount (mg)	% Recovery*	Manufacturer (India)
I	500	498.93	99.786	Lupin pharmaceuticals
II	500	499.21	99.842	Sigma pharma laboratories

*Mean of three replicates

Table 9: Assay of Flucytosine capsules.

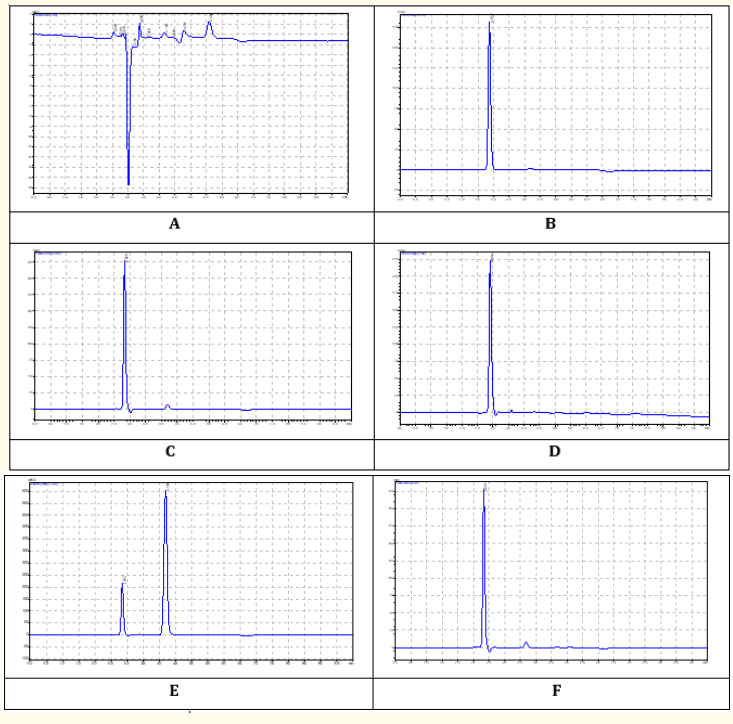

Figure 3: Typical chromatograms of A) Mobile phase (Blank) B) Flucytosine API (10µg/mL) and observations recorded during C) Acidic degradation D) Alkaline degradation E) Oxidative degradation F) Thermal degradation.

Figure 4: 3D chromatogram of Flucytosine A) Brand I B) Brand II (C) Acidic degradation (D) Alkaline degradation (E) Oxidative degradation (F) Thermal degradation

Figure 5: Purity plots of Flucytosine during degradation study.

Figure 6A: Overlay chromatograms of Flucytosine a) Blank b) Flucytosine Pure (10µg/mL) c) Acidic degradation d) Alkaline degradation e) Oxidation degradation f) Thermal degradation

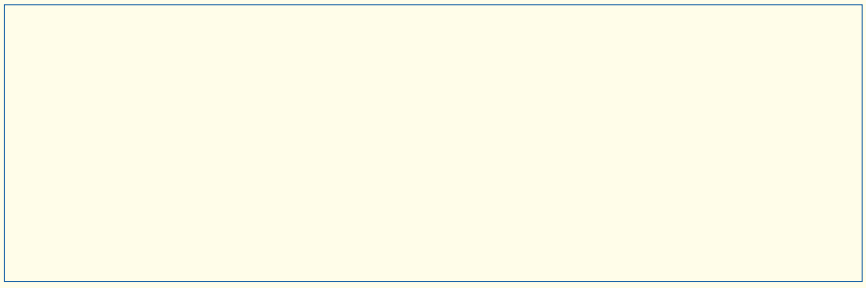

Figure 6B: Overlay chromatogram of a) Blank b) Flucytosine standard (10 µg/mL) c) Flucytosine capsules (Brand I) d) Flucytosine capsules (Brand II)

Assay of Flucytosine capsules

Assay was performed by using two brands of Flucytosine capsules consisting of 500 mg API and then it was found that the amount of Flucytosine was found to be 99.786-99.842 (Table 9) and there is no interference of excipients (Figure 6B)..

Conclusion

The RP-UFLC techniques were validated as per ICH guidelines and found to be simple, economical, simple and robust for the quantification of Flucytosine capsules.

Acknowledgement

The authors are grateful to M/s GITAM (Deemed to be University), Visakhapatnam for providing the research facilities and Lupin Pharmaceuticals (India) for supplying gift samples of Flucytosine. The authors have no conflict of interest.

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