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Development and validation of a stability indicating RP-HPLC method for determination of flucytosine and its process related impurities in injectable pharmaceuticals

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Abstract

A simple, sensitive, reproducible and cost effective reversed-phase liquid chromatography (RPHPLC) method coupled with a photodiode array detector was developed and validated for determination of Flucytosine and its related substances in pharmaceutical dosage forms, especially for injectable solution. The separation was achieved from octadecylsilyl silica gel, C18 (4.6 mm x 250 mm, 5µ) column with a mobile phase consisting of HPLC grade Water and Methanol (95:5) at a flow rate of 1ml/min with UV detection at 260 nm at 30°C column temperature. Total run time was 10 min within which main compound and other known (Fluorouracil) and unknown impurities were separated. Stability indicating capability was established by force degradation experiments and separation of known degradation products. This chromatographic method was optimized using the samples generated from forced degradation studies and the impurity spiked solution. Good resolution between the peaks corresponds to process-related impurities and degradation products from the analyte were achieved. The method was validated for Accuracy, Repeatability, Reproducibility and Robustness, Linearity, LOQ, LOD were established for Flucytosine and its impurities in a single RPHPLC method. Therefore, this method can be used as a more convenient and efficient option for the analysis of Flucytosine assay and it's related substances in injectable pharmaceutical dosage form to establish the quality of the drug product during routine analysis with consistent and reproducible results.

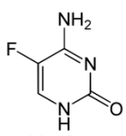
Keywords: Injectable pharmaceuticals, reversed-phase liquid chromatography; cytosine deaminase; 5-fluorocytosine.

1.Introduction

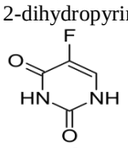
Flucytosine, a fluorinated pyrimidine analogue, is a synthetic antimycotic drug. Chemically it is referred to as 4-amino-5-fluoro-2(1H) - pyrimidinone. Flucytosine is structured like cytostatic fluorouracil and also resembles floxuridine[1][4]. 5-Fluorocytosine (5-FC; flucytosine) is a fluoropyrimidine that is used primarily in the management of systemic mycoses, such as candidiasis, chromoblastomycosis, cryptococcal meningitis, and torulopsosis[14].

5-Fluorouracil is used for the treatment of different cancer especially for outside layer of the adrenal gland, anus, bladder, cervix, endometrium, ovaries, esophagus, head and neck, penis, liver, prostate, skin, vulva, Carcinoid tumors[14].

Figure-01: Structure



4-amino-5-fluoro-1, 2-dihydropyrimidin-2-one (5-FC)



5-Fluoro-1*H*, 3H-pyrimidine-2, 4-dione (5-FU)

5- Fluorocytosine is very much stable in intravenous solution at regular storing temperatures and can therefore be stored at ambient temperatures for several years before the critical border of 95% 5-FC is reached. However, the toxic and teratogen degradation product 5-Fluorouracil (Specific impurity of 5-FC) may be present in the product, due to both impurities in the raw material and the formation from 5-FC upon sterilization and storage[5]. Identification and quantification of this impurity is very much important for pharmaceutical dosage forms especially for intravenous infusion.

Literature survey reveals that there are several studies found on determination of Flucytosine and also its metabolites in Plasma and other studies related to antifungal effect on different microorganisms or cultured cells[5][11], but there is no sensitive, cost effective, rapid, novel HPLC method and validation article to quantify the Pharmaceutical dosage forms especially for the injectable dosage forms. There is no compendial method present right now for injectable pharmaceuticals, although, there is method present for API or other dosage form but the related substances method is under thin layer chromatography (TLC) procedure. Related substances should be quantified in injectable pharmaceuticals which are directly bioavailable in the blood serum. Flucytosine produces Fluorouracil during sterilization[6]. 5-Fluorocytosine is deaminated by cytosine deaminase and produce 5-Fluorouracil and finally inhibition of protein synthesis and DNA synthesis occurred[12]. There is a study, which concludes with the suggestion to take sharp notice of the amount of the toxic and teratogen 5-FU in the product[1][6]. Systemic 5-fluorouracil can cause a syndrome of delayed myelin destruction in the central nervous system[13]. That's why quantification of its degrading products (Fluorouracil) is very important during formulation of injectable pharmaceutical dosage form. This study will confirm to determine Flucytosine and its degradents. Though this is a non compendial method for intravenous injection, it was validated in compliance with ICH Guidelines[15]-[16]. The present study describes an HPLC method, with a high sensitivity, precision and accuracy for determination of Flucytosine and its impurities in injectable pharmaceuticals. The objective of the study is to outline a easy and cost effective at the same time regulatory compliant method by which the identification and quantification of Flucytosine and its impurities can be introduced.

2. Materials and method

2.1 Reagents and Chemicals

All the solvents and chemicals used were of HPLC and analytical grade. Milli-Q water and 0.45

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µm Teflon filter was used throughout the experimental work. HPLC grade Methanol from (Merck, Germany); Flucytosine USP working standard (WS) was obtained from Nantong Jinghua Pharmaceutical Co, Ltd., China. Fluorouracil EPCRS from EP commission was used. Product development batches of Flucytosine IV infusion samples were provided as a gift from Square Pharmaceuticals Ltd., Bangladesh. Purified water was used for the analytical purpose.

2.2 Instruments

A Waters alliance, model-e2695 separation Module, USA equipped with a Waters 2998 Photodiode Array Detector (PDA) was used in the study. The HPLC method uses a Column: Hichrom 5 C18, 4.6 x 250 mm, 5µm, Data was recorded by using Empower software. Analytical balance from Mettler, pH meter from Metrohm, Switzerland, Ultrasonic bath from Sonoswiss from Switzerland, Water bath from Memmert, Germany were used.

2.3 Method Development

2.3.1 Chromatographic conditions

Different mobile phases were tested in order to find the best conditions for the separation of Flucytosine in presence of its potential impurity (Fluorouracil) and degradation products. The optimum composition of mobile phase was determined to be Water: Methanol (95:5).

In this HPLC method, we used octadecylsilyl silica gel, C18 (4.6 mm x 250 mm, 5µ) column, injection volume 20µl. Detection was carried out at 260 nm and the flow rate was 1.0 ml/min and the column temperature was 30°C.

2.4 Analytical Solutions

2.4.1 Preparation of standard solution

50.0 mg of Flucytosine standard was taken into a 100 ml volumetric flask. 60 ml Purified water was added and dissolved with help of ultrasonic bath for 10 minutes and mix thoroughly until it dissolves and then volume with purified water up to 100 ml and mix. 10 ml of this solution was diluted to 100 ml with Purified water and mix well. Final concentration set for 0.05 mg/ml.

2.4.2 System Suitability Solution

Weigh accurately about 5.0 mg of Fluorouracil CRS and transfer it into 100 ml Clean and dried volumetric flask. Add 50-60 ml of Purified water and sonicate for 10 minutes and mix thoroughly until it dissolves, and then volume with Purified water up to 100 ml and mix. Take 10 ml of this solution and 10 ml of the standard solution into a 50 ml clean and dried volumetric flask, then volume with Purified water up to 50 ml and mix well.

Final concentration for Flucytosine and fluorouracil was 0.01 mg/ml respectively.

2.4.3 Preparation of Sample solution

Pipette 5 ml of Flucytosine intravenous infusion into a 100 ml Clean and dried volumetric flask, add 50–60 ml of purified water & shake. Make volume up to 100 ml with the same and mix well. Concentration of this solution was 0.5 mg/ml. This solution will be used for Test solution for determination of known impurity (Fluorouracil) and any other unknown impurities.

10 ml of this solution was diluted to 100 ml with purified water and mix well for assay preparation for determination of Flucytosine (0.05 mg/ml). All solution was filtered with 0.20 µm membrane disk filters (PALL).

2.5 Method Validation

2.5.1 Results of forced degradation studies (Specificity)

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurity. The specificity of the developed HPLC method for Flucytosine was carried out in the presence of its impurity namely Fluorouracil. Stress studies were performed for Flucytosine bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of sunlight exposure and oxidation (10.0 % H2O2) and Thermal degradation was carried out heat at 80°C and was taken sample after 2 hours, 4 hours, 6 hours and 8 hours to evaluate the ability of the proposed method to separate Flucytosine from its degradation products. Peak purity test was carried out of Flucytosine peak by using PDA detector in stress samples. Assay studies were carried out of stress samples against qualified Flucytosine reference standard. Assay was also calculated for Flucytosine samples by spiking Fluorouracil at the specification level (i.e., 0.5%).

2.5.2 Precision

The precision of the assay method was evaluated by carrying out six independent assays of Flucytosine test samples against a qualified working standard and calculate the % R.S.D of assay. The precision of the related substances method was checked by injecting six individual preparations of Flucytosine (0.5 mg mL-1) spiked with 0.5 % Fluorouracil with respect to Flucytosine analyte concentration. % R.S.D of area for Fluorouracil was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

2.5.3 Limit of detection (LOD) and Limit of Quantification (LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitavely determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of Impurities and/or degradation products. The limit of detection (LOD) and limit of quantitation (LOQ) were separately determined at a signal to noise ratio (S/N) of 3 and 10.

2.5.4 Linearity

Linearity test solutions for the assay method were prepared from Flucytosine stock solutions at six concentration levels from 10% to 150% of assay analyte concentration (10%, 30%, 50%, 80%, 100%, 120%, 150%). The peak area verses concentration data was treated by least squares linear regression analysis. Linearity test solutions for the related substance method were prepared by dilution of stock solution to the required concentrations. The solutions were prepared at eleven concentration levels from LOQ to 150% of specification level (1.0%, 2%, 5%, 8%, 10%, 20%, 50%, 80%, 100%, 120% and 150%)for specific impurity (Fluorouracil) and thirteen concentration levels from LOQ to 1% of specification level (0.008%, 0.01%, 0.02%, 0.04 %, 0.06%, 0.08%, 0.1%, 0.2%, 0.4%, 0.5%, 0.6%, 0.8% and 1.0%) for Flucytosine. Above test were carried out of 3 consecutive days for Assay and 2 consecutive days for related substances method. The % RSD value for the Slope and Y-intercept of the calibration curve was calculated.

2.5.5 Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels 80%,100% and 120 % of sample solution concentration (0.05 mg mL-1) .The percentage of recoveries was calculated from the Slope and Y-

intercept of the calibration curve obtained in the linearity study. The accuracy study of impurities was carried out in triplicate at 80%, 100%, 120 % of specification level (0.5%) to the Flucytosine analyte concentration (50µg mL-1). The percentages of recoveries for impurities were calculated from the slope and Y- Intercept of the calibration curve.

2.5.6 Range

Data generated in linearity, precision and accuracy was considered for establishing the range of the analytical method.

2.5.7 Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Flucytosine and specific impurity (Fluorouracil) was recorded. The flow rate of the mobile phase was 1.0mL min-1.To study the effect of flow rate on the resolution, flow was changed by 0.2 units from 0.8 to 1.2 mL min-1. The effect of the column temperature on resolution was studied at 25°C and 35°C instead of 30°C. The effect of the percentage organic strength on resolution was studied by varying Methanol by -5 to + 5 % while other mobile phase components were held constant as stated in Chromatographic conditions.

2.5.8 Solution stability

The solution stability of Flucytosine in the assay method was carried out by leaving both the solutions of sample and reference standard in tightly capped volumetric flasks at room temperature and at 4°C for 48 hours. The solution stability of Flucytosine and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flasks at room temperature for 48 hours. Content of specific impurity (Fluorouracil) was determined after 48 hours. The mobile phase stability was also carried out for 48 hours by injecting the freshly prepared sample solutions. Content specific impurity (Fluorouracil) was checked in the test solutions.

3. Results and Discussion

3.1 Optimization of Chromatographic conditions:

The main objective of chromatographic method is to separate Flucytosine from specific impurity (Fluorouracil). The chromatographic separation was achieved on an Hichrom C18, 250 mm X 4.6 mm I.D with 5µ particles column, using mixture of Water and Methanol (95:5v/v) as a mobile phase. The flow rate of the mobile phase was 1.0 mL min-1, at 30°C column temperature, the peak shape of the Flucytosine was found to be symmetrical. In optimized chromatographic conditions of Flucytosine and specific impurity (Fluorouracil) separation was achieved with resolution greater than 4 and retention times were about 4.0 and 4.8 (Figure 6).

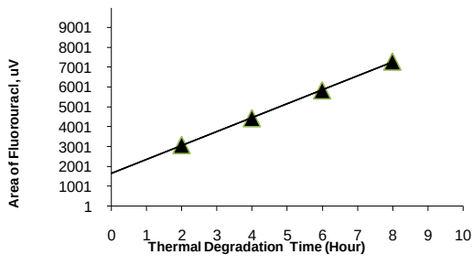
3.2 Results of forced degradation studies (Specificity)

Degradation was not observed in Flucytosine sample when subjected to stress conditions like sunlight. Significant degradation was noted in thermal study. Degradation was observed in oxidative conditions and in thermal degradation at 80°C for 2, 4, 6 and 8 hour's interval (Figure 7). Table-1 shows the degradation of Flucytosine by 80°C at analytical concentration and produce specific impurity (Fluorouracil) which indicates a linear degradation at a linear Time interval (2, 4, 6, 8 Hours of study). Thus during product development, it is very much important to set the sterilization temperature to maintain the limit of specific impurity (fluorouracil) under the limit stated for finished product. Peak purity test results confirmed that the Flucytosine peak is homogenous in stress samples.

Table 1: Linearity Degradation of Flucytosine by Thermal Degradation Study

Thermal Degradation study	
Hours	**Peak Area (Specific Impurity (Fluorouracil)**
2	3082
4	4442
6	5838
8	7288
Slope	700.7
y-intercept	1659
Correlation coefficient, r	0.9999

Figure 2: Linearity Degradation of Flucytosine by Thermal Degradation Study



3.3 Precision

System Precision was performed by replicate injections (n=6) of the standard solution at 100% of the sample concentration and calculating the % RSD of the measured area, theoretical plates and tailing factor and the Resolution solution of Flucytosine and Fluorouracil. Table-2 shows the Resolution Solution Data which indicates the resolution is greater than 4.0 and Relative retention time is 1.2. Table-3 shows the system precision data from Standard solution. From the data it was observed that the % RSD of Retention time and Area was 0.020 and 0.223 respectively which was well within the acceptance limit of 2.0%. Hence the system was precise.

Table 2: System Suitability Study-01

Peak Name.	Retention Time	RRT	Resolution	Tailing Factor	Theoretical Plate
Flucytosine	4.041	---	---	1.42	16937
Fluorouracil	4.781	1.2	5.85	1.29	20213

Table 3: System Suitability Study-02

Determination	Retention Time	Area	Tailing Factor	Theoretical Plate
1	4.040	1926190	1.44	16520
2	4.041	1924821	1.41	17265
3	4.040	1931668	1.43	16818
4	4.039	1926977	1.41	17293
5	4.040	1935910	1.43	16773
6	4.041	1932241	1.41	17158
Mean (n=6)	4.040	1929634	1.42	16971
Standard Deviation	0.001	4299.012	---	---
% Relative Standard Deviation (RSD)	0.020	0.223	---	---

The %RSD of assay of Flucytosine during the assay method precision study was 0.60% and the %RSD for the area of Flucytosine and Specific impurity (Fluorouracil) in related substances method precision study was within 1.0 %. (Table 4-5)

Table 4: Method Precision of Flucytosine in the Assay method Validation

Sample Number	Theoretical conc. of Flucytosine (mg/ml)	Determined conc. of Flucytosine (mg/ml)	% of Recovery
Sample-1	0.0504	0.0509	101.12
Sample-2	0.0504	0.0503	99.97
Sample-3	0.0504	0.0505	100.37
Sample-4	0.0504	0.0510	101.25
Sample-5	0.0504	0.0510	101.23
Sample-6	0.0504	0.0511	101.41
Mean (%)			100.89
Standard Deviation			0.58
% Relative Standard Deviation			0.57

Table 5: Spiked Sample Method Precision Recovery for Related Substances

Sample Number	Determined conc. of Fluorouracil (ppm)	Area	% of Recovery
Sample-1	2.72120	163369	101.16
Sample-2	2.71959	163273	101.10
Sample-3	2.72820	163779	101.42
Sample-4	2.73465	164171	101.66
Sample-5	2.72981	163878	101.48
Sample-6	2.72847	163798	101.43
Mean (%)			101.38
Standard Deviation			0.209
% Relative Standard Deviation			0.207

Sample Number	Determined conc. of Flucytosine (ppm)	Area	% of Recovery
Sample-1	0.9992	40546	99.56
Sample-2	1.0106	41011	100.70
Sample-3	0.9909	40210	98.73
Sample-4	0.9896	40157	98.60
Sample-5	0.9861	40020	98.26
Sample-6	0.9928	40289	98.92
Mean (%)			99.13
Standard Deviation			0.882
% Relative Standard Deviation			0.890

3.4 Limit of detection (LOD) and Limit of Quantification (LOQ)

The limit of detection of specific impurity (Fluorouracil) and Flucytosine was achieved at 0.01µg mL^{-1} each for 20 µL injection volume. The limit of quantification of Flucytosine and specific impurity (Fluorouracil) is 0.025 µg mL-1 and 0.04 µg mL-1. Table 6 shows the precision at the LOQ concentrations for Flucytosine and Fluorouracil at 0.2% and 0.5% of specification level to Flucytosine analyte concentration respectively and %RSD obtained was 1.99% and 1.60% respectively.

It is also based on signal-to-noise ratio and it was observed that the Signal –to- Noise ratio of Flucytosine is 12.0 at 0.04 ppm and 11.1 for Fluorouracil at 0.025 ppm at 0.2% and 0.5% of specification level to Flucytosine Analyte Concentration 0.5mg/ml. So the quantitation limit was established as 0.04 ppm (0.008%) for Flucytosine and 0.025 ppm (1.0%) for Fluorouracil.

Table 6: LOQ precision of Flucytosine and Fluorouracil

	Injection No.	Peak area of Flucytosine	Signal to noise ratio of Flucytosine peak	Peak area of Fluorouracil	Signal to noise ratio of Fluorouracil peak
LOQ Concentration: 0.04 ppm (Flucytosine) and 0.025 ppm (Fluorouracil)	1	1680	11.5	1709	10.2
	2	1738	11.5	1739	10.0
	3	1748	13.8	1691	12.1
	4	1737	12.3	1680	10.7
	5	1700	11.0	1711	12.1
	6	1776	11.9	1661	11.2
Mean		1729	12.0	1698	11.1
Standard Deviation		34.50	NA	27.24	NA
% Relative Standard Deviation		1.99	NA	1.60	NA

3.5 Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 5–75 µg mL-1 and correlation coefficient obtained was greater than 0.999. Table-7 shows the result that an excellent correlation existed between the peak area and concentration of the analysis. Linear calibration plot for the related substances method was obtained over the calibration ranges tested i.e. 0.008% to 1.0% for Flucytosine and 1% to 150.0 % for specific impurity (Fluorouracil) spiked with 0.2% of Flucytosine and 0.5 % Fluorouracil respect to Flucytosine analyte concentration (0.5 mg mL-1). Table, 8-9 shows the correlation coefficient obtained greater than 0.99. Linearity was checked for the related substances method over the same concentration ranges. The above results shown that an excellent correlation existed between the peak areas and the concentrations of Flucytosine and Fluorouracil.

Table 7: Linearity test of Flucytosine Standard solution

% Level	Concentration (%)	Peak Area
10	10	200259
30	30	577312
50	50	966184
80	80	1527238
100	100	1894551
120	120	2284420
150	150	2859224
Slope		18953
y-intercept		10670
Correlation coefficient, r		1.000

Figure 3: Linearity test of Flucytosine Standard solution

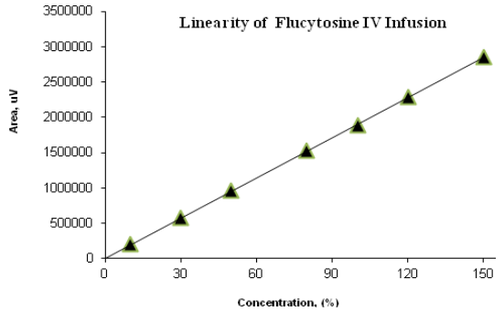


Table 8: Linearity for Flucytosine (0.2% of Analyte concentration 0.5 mg/ml)

Level	Concentration (ppm)	Nominal Conc (%)	Peak Area
1	0.040	0.008	1769
2	0.050	0.01	2159
3	0.100	0.02	4048
4	0.200	0.04	8065
5	0.300	0.06	11993
6	0.400	0.08	16080
7	0.500	0.1	20397
8	1.000	0.2	40813
9	2.000	0.4	81097
10	3.000	0.6	112970
11	4.000	0.8	150743
12	5.000	1.0	187500
Correlation coefficient			0.9996
SLOPE			40569
y-intercept			1288.4
LOD (ppm)			0.01
LOQ (ppm)			0.04

Figure 4: Linearity for Flucytosine (0.2% of Analyte concentration 0.5 mg/ml)

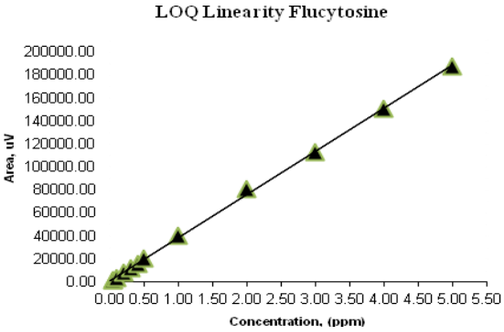
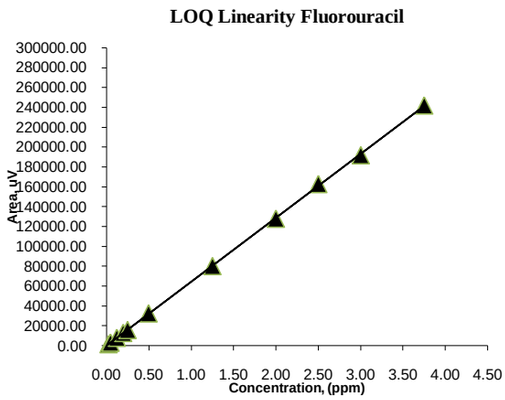


Table 9: Linearity for Fluorouracil (0.5% of Analyte concentration 0.5 mg/ml)

Level	Conc. (ppm)	Nominal Conc. (%)	Peak Area
1	0.0320	5	5955
2	0.025	1	1530
3	0.050	2	3197
4	0.125	5	7986
5	0.200	8	13023
6	0.250	10	16087
7	0.500	20	32533
8	1.250	50	80106
9	2.000	80	128017
10	2.500	100	162903
11	3.000	120	191604
12	3.750	150	242018
Correlation coefficient			0.99995
y-intercept			14.264
SLOPE			64005
LOD (ppm)			0.01
LOQ (ppm)			0.025

Figure 5: Linearity for Fluorouracil (0.5% of Analyte concentration 0.5 mg/ml)



3.6 Accuracy

The percentage recovery of Flucytosine in bulk drug samples was ranged from 99.5 to 100.15**%**.The percentage recovery of Flucytosine (0.2% of specification level to Flucytosine Analyte Concentration 0.5 mg/ml) varied from 99.35% to 101.25% and specific impurity i.e. Fluorouracil (0.2% of specification level to Flucytosine Analyte Concentration 0.5 mg/ml) in Flucytosine IV Infusion varied from 98.78% to 101.13%. (Table 10-12). The HPLC chromatograms of unspike standard solution, spike sample of Flucytosine and Fluorouracil at 0.2% and 0.5% of specification level to Flucytosine Analyte concentration (0.5 $mgml^{-1}$) respectively in bulk drug samples are shown in Figure 8.

Table 10: Accuracy of Flucytosine (Assay)

Sample No	Spiked level (%)	Theoretical conc. of Flucytosine (mg/ml)	Determined conc. of Flucytosine (mg/ml)	% of Recovery	Mean (%) and RSD
1	80	0.04020	0.0399	100.33	100.07 0.08
2	80	0.04020	0.0399	100.04	
3	80	0.04020	0.0398	100.07	
4	100	0.05025	0.0496	99.63	99.55 0.09
5	100	0.05025	0.0493	99.56	
6	100	0.05025	0.0497	99.45	
7	120	0.06030	0.0603	100.00	100.15 0.16
8	120	0.06030	0.0603	100.05	
9	120	0.06030	0.0604	100.16	
Grand average (%)					99.92
Pooled RSD					0.326

Table 11: Accuracy of Flucytosine (0.2% of specification level to Flucytosine Analyte Concentration)

Sample No	Spiked level (%)	Theoretical conc. of Flucytosine (ppm)	Determined conc. of Flucytosine (ppm)	% of Recovery	Mean (%) and RSD
1	50	0.5	0.5010	100.20	99.79 0.37
2	50	0.5	0.4985	99.69	
3	50	0.5	0.4974	99.48	
4	100	1.0	1.0258	102.58	101.25 1.68
5	100	1.0	0.9933	99.33	
6	100	1.0	1.0183	101.83	
7	200	2.0	2.0144	100.72	99.35 1.59
8	200	2.0	1.9938	99.69	
9	200	2.0	1.9524	97.62	
Grand average (%)					100.13
Pooled RSD					1.35

Table 12: Accuracy of Fluorouracil (0.5% of specification level to Flucytosine Analyte Concentration)

Sample No	Spiked level (%)	Theoretical conc. of Fluorouracil (ppm)	Determined conc. of Fluorouracil (ppm)	% of Recovery	Mean and % RSD
1	80	2.0	1.9914	99.57	99.64 0.059
2	80	2.0	1.9936	99.68	
3	80	2.0	1.9932	99.66	
4	100	2.5	2.5235	100.94	101.13 0.18
5	100	2.5	2.5288	101.15	
6	100	2.5	2.5328	101.31	
7	120	3.0	2.9403	98.01	98.78 0.679
8	120	3.0	2.9742	99.14	
9	120	3.0	2.9760	99.20	
Grand average (%)					99.85
Pooled RSD					0.41

3.7 Range

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It will be established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, precision when applied to samples containing amounts of analyte within the extremes of the specified range of the analytical procedure. The minimum specified range should be considered for the assay of Flucytosine normally from 80 to 120 percent of the test concentration (0.05 mg/ml) and for Relative substances method, 80% to 120% and 0.1% to 0.4% of specification level to Flucytosine Analyte Concentration for Fluorouracil and Flucytosine respectively. (Table: 13-14)

Table 13: Range Study (Assay Method)

Parameter	Concentration Range	Acceptance Limit	Result
Linearity	10% to 150%	R NLT 0.995	R= 1.0000
Method Precision	100% of Sample Solution	% RSD = NMT 2.0 Assay 98% to 102%	% RSD is 0.32%, Assay 99.85% to 100.57%
Intermediate Precision	100% of Sample Solution	% RSD of two analyst NMT 2.0, Assay 98% to 102%, %RSD of Assay NMT 2.0	% RSD of two analyst 0.67, Assay: 99.83.% – 100.10%, % RSD of Assay: 0.11
Accuracy	80% to 120% of Sample solution Concentration.	%Recovered= 98 % to 102%	99.55 -100.15

Table 14: Range Study (Related Substances Method)

Parameter	Concentration Range	Acceptance Limit	Result
Linearity at LOQ concentration	0.008% to 1% for Flucytosine at LOQ level 1% to 150% for Fluorouracil at LOQ Level	R NLT 0.99	**Flucytosine:** 0.9996 **Fluorouracil:** 0.99995
Method Precision	100% and 0.2% of specification level to Flucytosine Analyte Concentration for Fluorouracil and Flucytosine respectively	% RSD = NMT 5.0 % Recover 90% to 110%	**Fluorouracil:** % RSD is 0.20%, Assay 101.10% to 101.66% & **Flucytosine:** % RSD is 0.89%, Assay 98.26% to 100.70%
Intermediate Precision	100% and 0.2% of specification level to Flucytosine Analyte Concentration for Fluorouracil and Flucytosine respectively	% RSD of two analyst NMT 2.0 Assay 90% to 110% %RSD of Assay NMT 2.0	**Fluorouracil:** % RSD of two analyst 0.50, Assay: 99.54.% – 102.57%, % RSD of Assay: 1.14 **Flucytosine:** % RSD of two analyst 0.78, Assay: 97.98.% – 101.60%, % RSD of Assay: 1.83
Accuracy	80% to 120% and 0.1% to 0.4% of specification level to Flucytosine Analyte Concentration for Fluorouracil and Flucytosine respectively	%Recovered= 98 % to 102%	**Fluorouracil:** 98.79 – 101.13 **Flucytosine:** 99.35 – 101.25

3.8 Robustness

In all the deliberate varied chromatographic conditions (flow rate, composition of organic solvent & column temperature) the resolution between critical pair, i.e. Flucytosine and Fluorouracil was greater than 3.5, illustrating the robustness of the method. (Table 15)

Table 15: Data of System suitability & % Assay (Robustness study) for Assay Method

Condition	Resolution	Area of Standard	%RSD of Area	% Recovery Acceptance Limit AMV: 98.0% to 102.0% RSMV: 90.0% to 110.0%
Analyst 1	4.61	1942112		100.89
Analyst 2	5.82	1952057	0.15	99.93
Temperature 25°C	5.55	1937803	0.58	99.14
Temperature 35°C	4.69	1932078	0.08	99.20
Flow Rate 0.8 ml/min	5.30	2404806	0.12	99.55
Flow Rate 1.2 ml/min	6.36	1603728	0.15	99.19
Organic + PW (10+90)	3.78	1928106	0.32	100.19
Organic + PW (0+100)	7.30	1925538	0.20	100.17
Mean %				99.78
Standard Deviation				0.62
% Relative Standard Deviation				0.62

Data of System suitability & % Assay (Robustness study) for Related Substances Method

Condition	Resolution	Area of Standard	%RSD of Area	% Recovery Acceptance Limit 90.0% to 110.0%	
Analyst 1	6.78	1917641	0.22	Flucytosine	99.15
				Fluorouracil	100.24
Analyst 2	5.90	1929918	0.12	Flucytosine	100.25
				Fluorouracil	99.53
Temperature 25°C	6.88	1928358	0.10	Flucytosine	99.20
				Fluorouracil	98.87
Temperature 35°C	5.96	1932417	0.37	Flucytosine	94.66
				Fluorouracil	98.67
Flow Rate 0.8 ml/min	6.71	2425472	0.17	Flucytosine	99.19
				Fluorouracil	99.45
Flow Rate 1.2 ml/min	6.36	1612275	0.08	Flucytosine	98.22
				Fluorouracil	99.26
Mean %				98.89	
Standard Deviation				1.45	
% Relative Standard Deviation				1.46	

3.9 Solution stability

The %RSD of assay of Flucytosine during solution stability experiment was within 0.2%. No significant changes were observed in the content of impurities namely specific impurity (Fluorouracil) during the solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phases used during the assay and the related substance determination were stable for 48 hours.

Table 16: Solution Stability:

Time Interval	% Assay	Difference in % Assay initial
Initial Standard	100.00	---
After 48 Hrs at RT Standard	100.14	0.14
After 48 Hrs at 2-8 Standard	100.16	0.16
Initial Sample	100.06	---
After 72 Hrs at RT Sample	99.63	0.43
After 72 Hrs at 2-8°C Sample	100.06	0.00
Mean %	100.00	
Standard Deviation	0.19	
% Relative Standard Deviation	0.19	

Figure 6: Representative Chromatogram of Flucytosine and specific impurity (Fluorouracil) at concentration 0.01 mg/ml respectively.

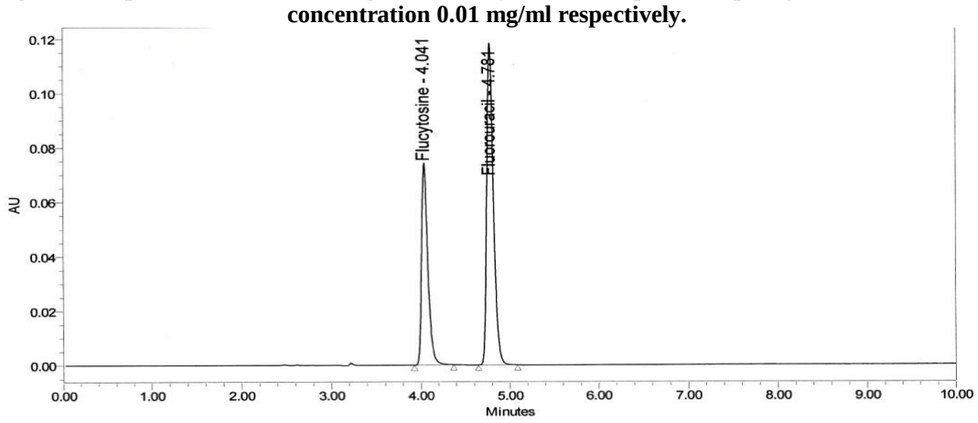
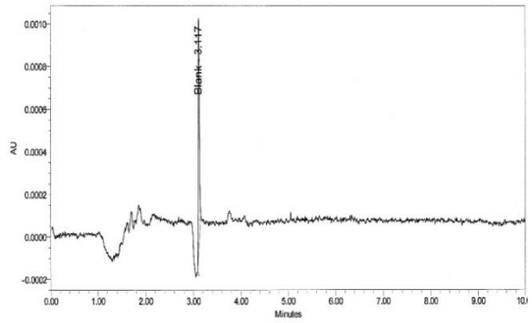
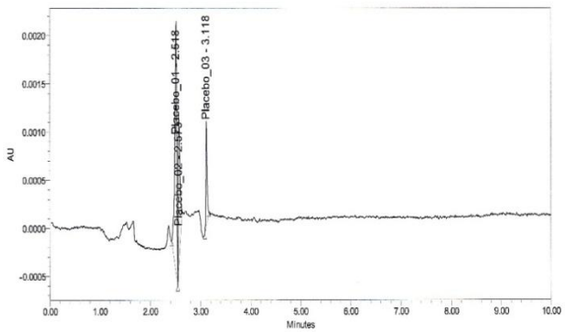


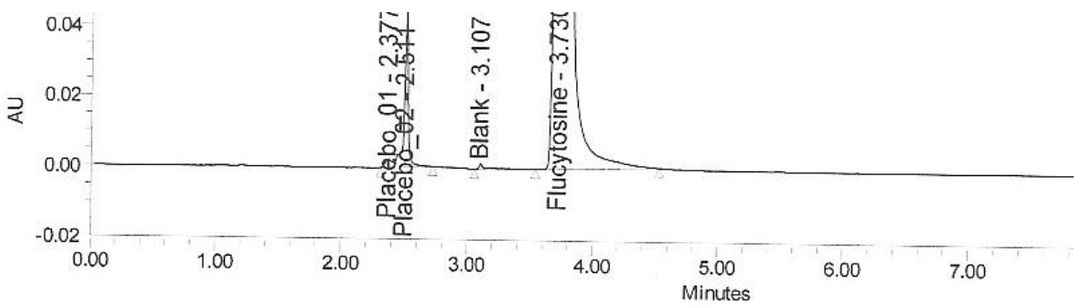
Figure 7: Thermal degradation at 80°C for 2, 4, 6 and 8 hour's interval



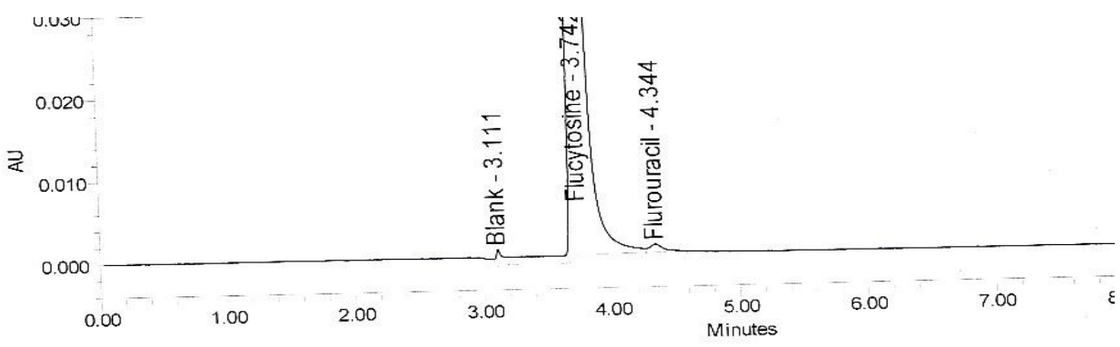
Blank



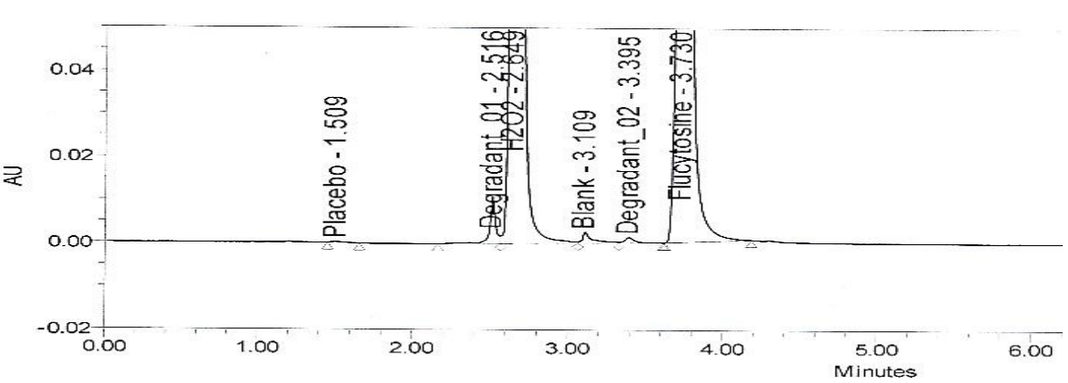
Placebo



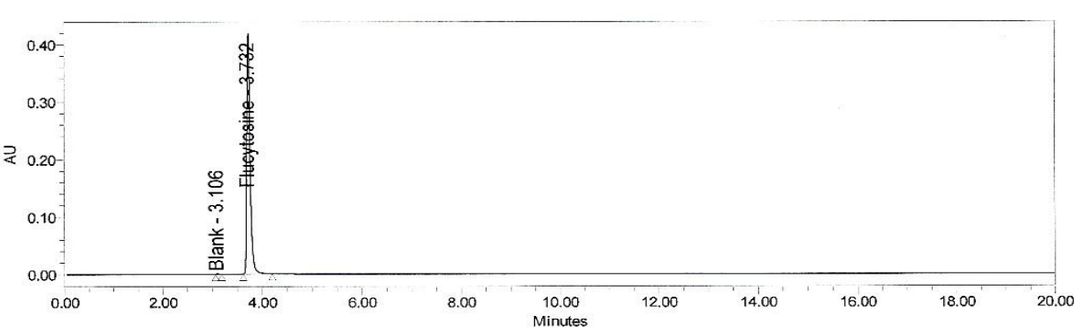
Control Sample



Thermal Degradation

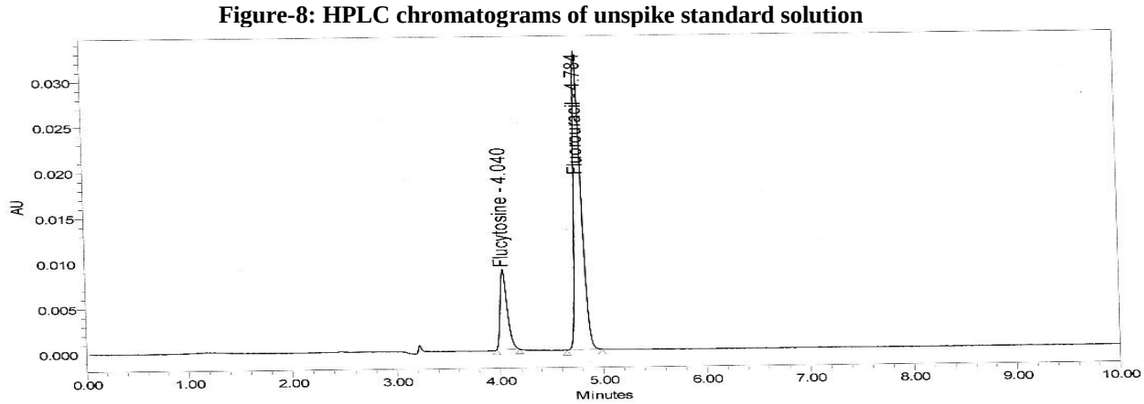


Peroxide Degradation

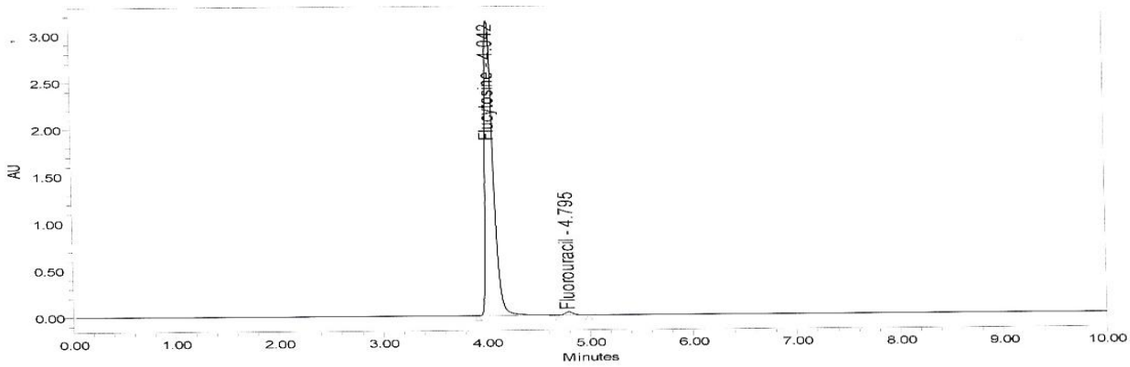


Sunlight Degradation

Figure-8: HPLC chromatograms of unspike standard solution



Flucytosine 0.2% + Fluorouracil 0.5% of specification level to Flucytosine Analyte concentration



Fluorouracil 0.5% of specification level to Flucytosine Analyte concentration (0.5 mg/ml)

4. Conclusion

The HPLC method developed for quantitative and related substance determination of Flucytosine is linear, accurate, precise, rapid and specific. The method was fully validated showing satisfactory data for all method validation parameters tested. The developed method is stability indicating and can be conveniently used for quality control to determine the related substance and assay in regular Flucytosine product development, production and stability samples.

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