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Research Article

Development and validation of stability indicating HPLC method for the estimation of 5-Fluorouracil and related substances in topical formulation

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ABSTRACT

A sensitive HPLC method has been developed and validated for the determination of 5-Fluorouracil (5-FU) and related substances in a novel topical formulation. The highly polar molecule requires aqueous mobile phase for the elution and separation of 5-Fluorouracil and its impurities (Impurity A, B and C official in EP). The developed method is found to be specific, reproducible, and stability indicating. The Phenomenex Synergi Polar RP 250×4.6mm 4µ column was used and mobile phase consisted of 0.1M potassium di hydrogen phosphate (KH_2PO_4) buffer to achieve good resolution and retention of the analyte and its impurities. The detector linearity was established from concentrations ranging from 0.01 µg/ml to 500 µg/ml for 5-Flurouracil and from 0.01 µg/ml to 0.08 µg/ml for related substances with a correlation co-efficient of 0.999. The relative response factor (RRF) values of impurity A, impurity B and impurity C determined from linearity plots were 1.9, 0.9 and 1.4 respectively. The limit of detection (LOD) and limit of quantification (LOQ) found to be in a range of 0.004 µg/ml and 0.014µg/ml for 5-Fluorouracil and related substances respectively. The molecule was stable in all the stress conditions such as acid, base, oxidation, heat and photolysis as per the recommendations of ICH guidelines. The method was proved to be robust with respect to changes in flow rate, pH and column temperature. The proposed method is found to be sensitive, precise, rapid, reproducible, and offers good column life.

Keywords: 5-Fluorouracil; HPLC method; Validation; Stability indicating; topical formulation.

1. INTRODUCTION

5-Fluorouracil is an anti cancer drug used in the topical therapy of skin cancer (Wilgus TA et al., 2004). It is also used to treat malignant tumors of liver, colon, breast and stomach (Schaaf LJ et al., 1978, Kirkwood JM et al., 1980). It is a pyrimidine analogue interrupts the action of enzyme thymidylate synthase there by inhibiting the synthesis of thymidine which is required for the replication of DNA (Noordhuis P et al., 2004). The drug is used in different formulations for the skin therapy. In the present study 5-FU in a novel topical formulation was used to evaluate the chromatographic separation of 5-Fluorouracil and its related impurities.

Literature reveals few RP-HPLC methods for determination of 5-Fluorouracil (Ibrahim A et al., 2004, Dafeng C et al., 2003, Arbos P et al., 2002, Sampson DC et al., 1982) in dosage forms. The reported methods found, lack in sensitivity and are not capable of producing proper resolution between uracil and 5-FU. Since the molecule is highly polar in nature, its retention and separation from the related substances in RP columns requires aqueous mobile phase. The use of such mobile phase in RP columns leads to poor retention and selectivity of analytes and hence poor reproducibility (Oslen BA et al., 2001). The polar stationary phases although enhances the retention of 5 FU but results in a poor column life (Kazoka H et al., 2003). The main objective is to develop and validate a simple, effective and reproducible HPLC method for the determination of 5-Fluorouracil and related substances in a novel topical formulation. Aqueous solution containing potassium di hydrogen phosphate buffer was used as mobile phase (Coe RA et al., 1996, European Pharmacopoeia).The Phenomenex Synergi Polar RP-80Å column was selected to enhance retention capacity, sensitivity and specificity of the analyte and its related substances.

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2. MATERIALS & METHODS

2.1. Materials & reagents

5-Fluorouracil (purity -100%) and the impurities of 5-Fluorouracil pyrimidine-2,4,6 (1H,3H,5H)-trione (impurity A), Dihydropyrimidine-2,4(3H)-trione (impurity B)

and Pyrimidine-2,4(1H,3H)-dione (impurity C) official in European Pharmacopoeia was obtained from LGC Promochem, Potassium di-hydrogen phosphate (AR grade- Merck (India) limited. All the other chemicals and solvents used were of analytical grade or HPLC grade.

2.2. Apparatus

The analysis was carried out on Waters Alliance HPLC systems 2695 separation module connected to 2996 Photo diode array detector. Data acquisition was carried out using Empower software.

Different chromatographic column used during trials were

1. Phenomenex Synergi- Polar RP 80A (250mm×4.6mm), 4µ (make-Phenomenex).
2. XTerra RP18 (250mm×4.6mm)5 µ, (make-Waters).
3. Waters Symmetry C18, (250×4.6mm), 5 µ, (make-Waters).
4. Hypersil BDS C18, 250×4.6mm, 5 µ, (make-Thermo-SCIETIFIC).
5. Inertsil ODS 3V, 250×4.6mm, 5 µ, (make-GLSciences).
6. XTerra RP 8, 250×4.6mm, 5 µ (make-Waters).
7. Hypersil BDS C8, 250×4.6mm, 5 µ , (make-Thermo-SCIETIFIC)

2.3 Chromatographic conditions

The separation of 5-Fluorouracil (5-FU) and related substances were achieved by using 0.1M potassium dihydrogen phosphate buffer, pH adjusted to 6.0 using 5M potassium hydroxide solution as mobile phase at a flow rate of 1.0ml/minute. Detection and purity establishment of the main drug and the related substances were achieved by using a photo diode array (PDA) detector at 266nm. The drug samples and formulation samples were prepared in mobile phase (diluent) to achieve a concentration of 100µg/ml and 20µl of the sample were injected. The run time optimized was found to be 20 minutes.

2.4 Standard preparation

Standard stock solution (100µg/ml) was prepared in mobile phase which is used as diluent. About 50mg of the working standard was transferred to 50ml volumetric flask, dissolved in diluent with sonication and diluted to volume, 5.0ml of the stock solution was pipetted to 50ml volumetric flask and diluted to volume with diluent to achieve a concentration of 100µg/ml. A system suitability test was performed for six replicate standard injections. The resolution solution was prepared by making a stock solution of each impurity in methanol with a concentration of about 100µg/ml and further diluting the impurities to 10 µg/ml in mobile phase.

2.5 Sample Preparation

The drug was extracted from the novel topical formulation of 5% (w/w) label claim by using the mobile phase as diluent (USP, Hus LSF et al., 1980). About 1g of the topical formulation was taken in 100ml stoppered conical flask, 80 ml of diluent was added and sonicated for 30 minutes and cooled to room temperature. The solution was transferred to 100ml volumetric flask and diluted to volume using diluent. The solution was further diluted by pipetting 10.0ml of the stock solution to 50ml volumetric flask, diluting it to volume using diluent to achieve a concentration of 100 µg/ml.

3. RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic conditions

Several columns were used for optimizing the chromatographic condition. The parameters being focused were improvisation of retention time of 5-Fluorouracil, separation of related impurities and column life. Though 5 -FU retained for longer time in silica column, the column get deteriorated faster because of 100% aqueous mobile phase (Coe RA et al., 1996). There was poor resolution of main peak with the impurity peaks and the peak shape was non Gaussian type in X-Terra RP 18 column. The symmetry C-18 and Hypersil BDS C-18 did not provide good peak shapes, peak splitting was observed with impurity B. In X-Terra RP-8 only 3 peaks were resolved out of four components injected. The Hypersil C-8 exhibited lower peak separation, peak

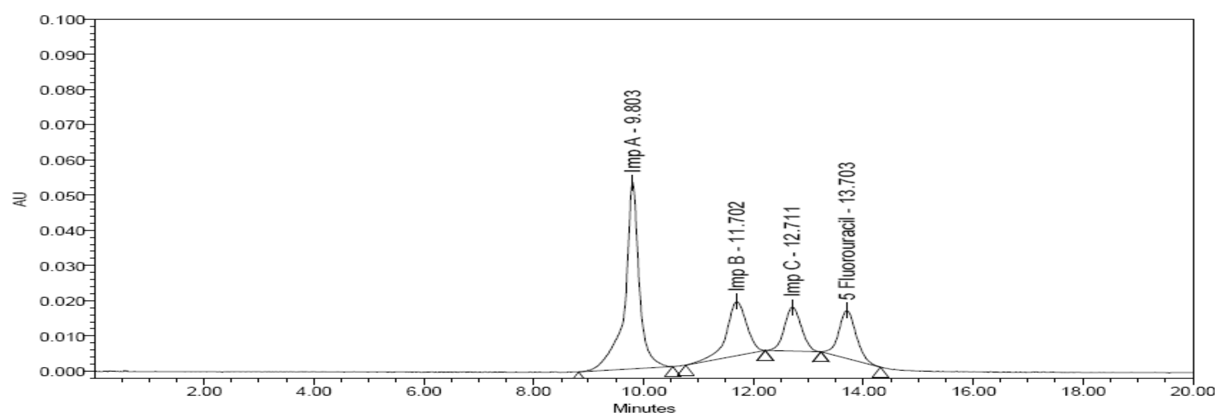

Figure 1: X-Terra RP18 250*4.6mm, 5µ

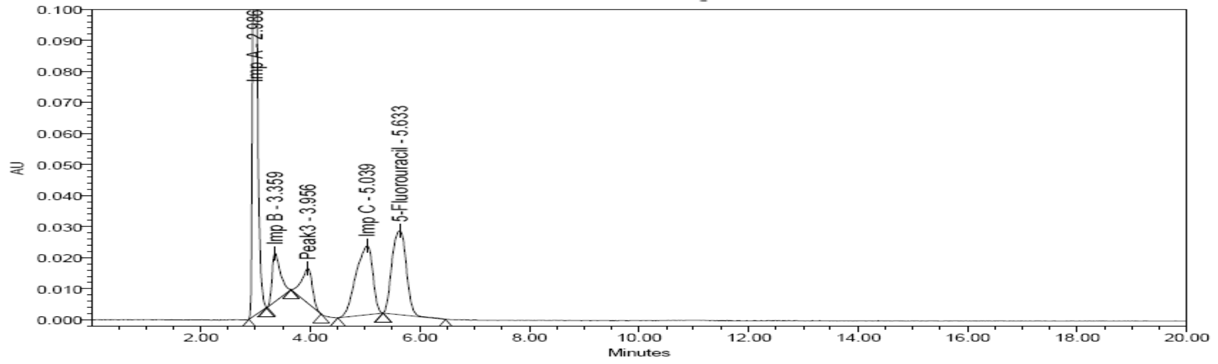

Figure 2: Hypersil BDS C18, 250*4.6mm, 5µ

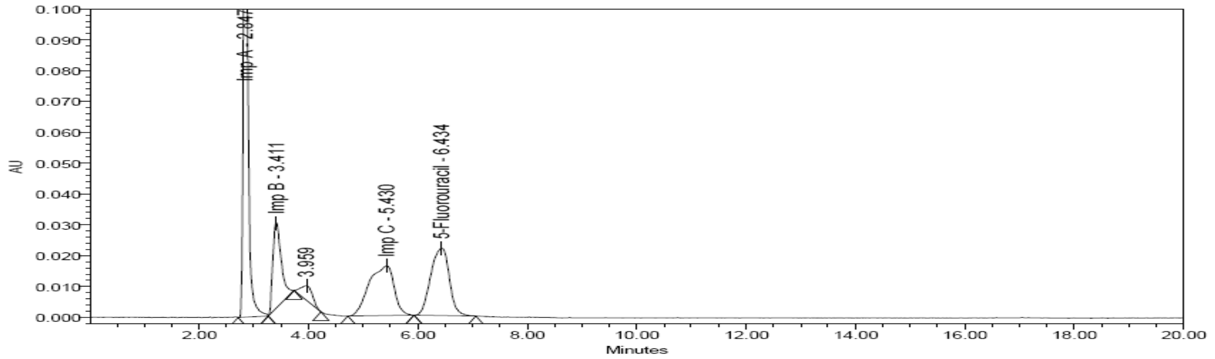

Figure 3: Waters Symmetry C18, 250*4.6mm, 5µ

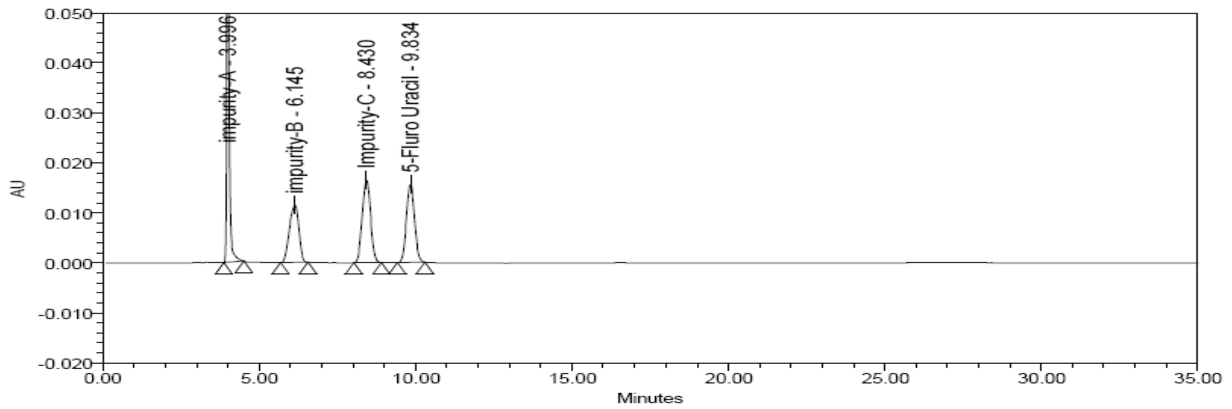

Figure 4: Inertsil ODS 3V, 250*4.6mm, 5µ

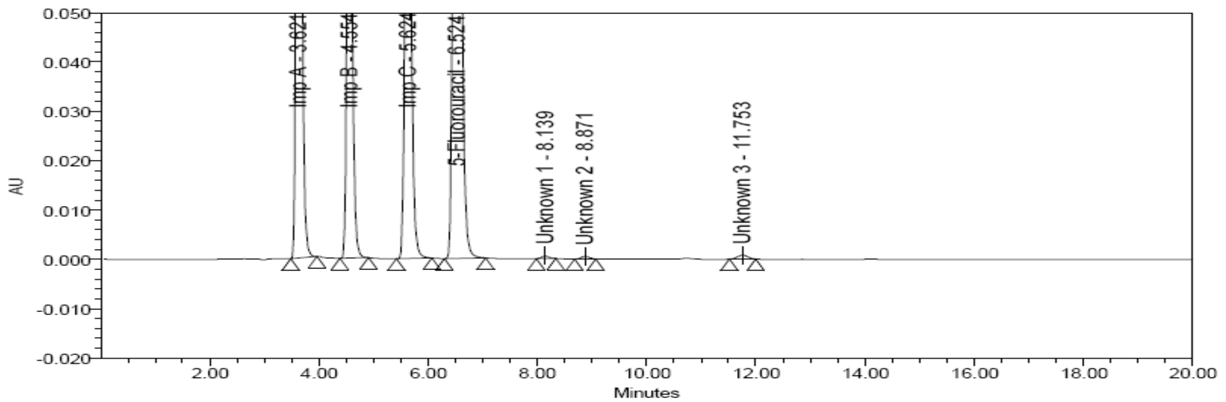

Figure 5: Chromatogram of Resolution solution containing 5 FU, Impurity A, Impurity B and Impurity C

splitting was observed for both impurity B, impurity C, and the 5-Fluorouracil peak was found to be very broad. Inertsil ODS 3V, 250×4.6mm, 5 µ offered good resolution of 5 FU and impurities but the LOD and LOQ concentrations were difficult to establish as the peak area response was not reproducible at lower concen-

tration levels. The phenomenex synergy polar RP 250x4.6mm, 4μ column was suitable to resolve 5 FU and all impurities within 18 minutes. The sensitivity of the method was increased with this column in comparison with Inertsil ODS 3V. Synergi polar-RP is an ether-linked phenyl phase with proprietary hydrophilic end-capping designed specifically to maximize retention and selectivity for polar and aromatic analytes. The ether-linkage present in the stationary phase is a polar embedded group offers improved peak shape and resolution of the highly polar compounds. Chromatograms in different columns were shown in figure 1, 2, 3, 4, and 5.

3.1.1. Buffer Selection.

Different buffers such as potassium phosphate, sodium phosphate and acetate were evaluated for system suitability parameters and overall chromatographic performance. In the sequential trials carried out using different buffers it was concluded that potassium di hydrogen phosphate was found to be suitable for effective separation of parent peak and impurities. Potassium di-hydrogen phosphate buffer pH 6.0 with different concentrations ranging from 0.05M, 0.1M and 0.2 M were tried. It was observed the change in buffer concentration did not offer significant changes in the elution pattern and resolution, but 0.1M concentration increased the sensitivity of method.

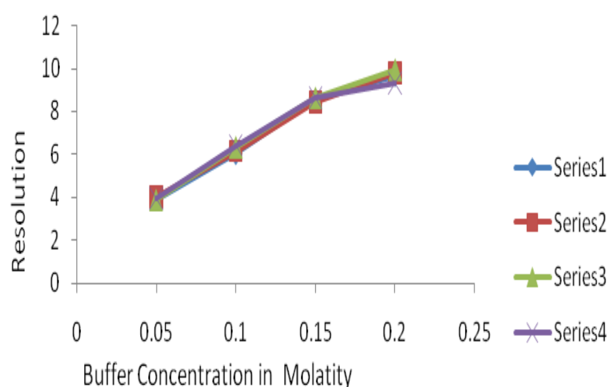

Figure 6: Effect of buffer concentration on resolution

3.1.2. Effect of pH.

The pH had no effect on the retention time of 5-FU and its related compounds but the peak asymmetry had shown some changes on alteration in the pH

Table 1: Effect of pH on peak symmetry

pH	A	B	C	5-FU
4	1.58	1.49	1.59	1.62
4.5	1.35	1.42	1.52	1.48
5	1.34	1.35	1.36	1.46
5.5	1.32	1.33	1.23	1.42
6	1.24	1.25	1.18	1.35
7	1.33	1.62	1.47	1.26

3.1.3. Effect of organic modifier

The usage of organic solvents like acetonitrile and methanol gave a poor chromatographic picture with poor resolution. Introduction of organic modifier has shown poor theoretical plate for different impurities.

3.2. Optimized Method

The chromatographic condition optimized were Synergi Polar RP 80Å 250x4.6mm- 4μ column with 0.1M KH_2PO_4 buffer pH 6.0. The retention time of 5-Fluorouracil, impurity A, impurity B and impurity C was found to be 3.6,4.5,5.8 and 6.6 minutes respectively. The chromatogram is shown in (figure 5). The relative retention time (RRT) of impurity A, impurity B and impurity C were found to be 0.55, 0.69 and 0.89 respectively with respect to the analyte peak. The method was capable of separating the impurities and the main drug with resolution not less than 4.2 between each peak. The tailing factor for all the impurity and main peak was found to be 1.0 and the theoretical plates were not less than 8000. The peak purity of all the impurities was passed and no flag in purity were observed. The purity curves for 5FU and all impurities are given in (figure 7 to figure 10). System suitability parameters are given in (tab. 3). The chromatographic conditions were suitable for both assay and related substances analysis as the method is highly sensitive.

3.3. Drug extraction from the formulations

The extraction of drug from the formulation matrix was tried using different solvents such as 100% water, mobile phase, water with methanol, acetonitrile and tetrahydrofuran. The complete extraction of drug was achieved in water and mobile phase. The addition of methanol in the diluent was avoided as literatures indicate that the drug adsorbs to the glass surface in the presence of methanol (Drissen O et al., 1978). The diluent containing tetrahydrofuran shown poor extraction from the matrix of in house formulation (hydrophilic gel based matrix) since the latter was hydrophilic in nature and the drug was completely soluble in 100% aqueous solution.

3.4. Validation of method

3.4.1. Specificity

The Forced degradation of API, placebo and formulation was carried out as per ICH guidelines (ICH Q_2B) in acid, base, oxidation, heat and photolysis. The acid, base and oxidation stress studies were carried out by refluxing API for 24 hours with 10ml 5N HCl, 5N NaOH and 30% hydrogen peroxide respectively. The thermal degradation was carried out by heating the drug powder at 105°C for about 24 hrs and the photo degradation was performed exposing the drug material to 1.2 million lux hours and 200 watt hours/M^2. The drug and the formulation were found to be stable under all the stress conditions. All the stress conditions with

purity angle and purity threshold are reported in table 2.

3.4.2. System suitability

The system suitability was checked by making five rep-

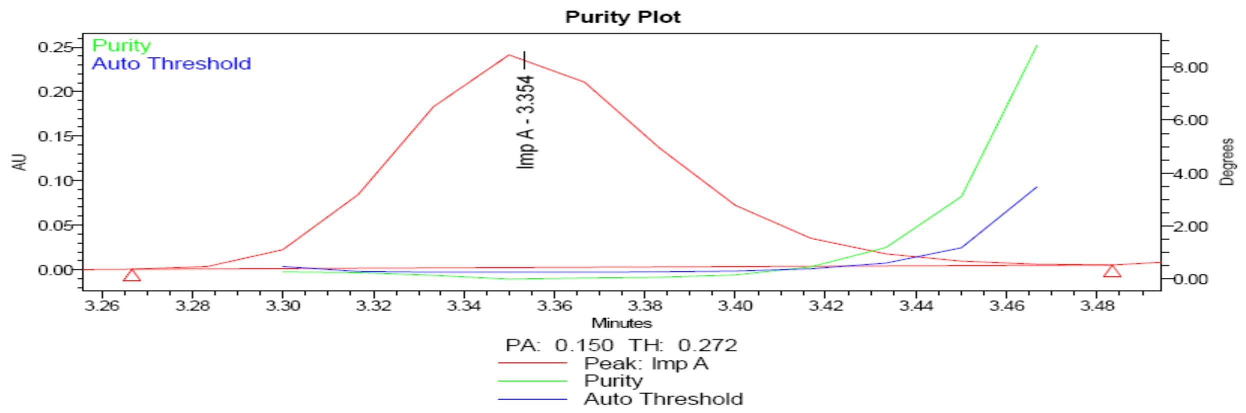

Figure 7: Peak purity of Impurity A

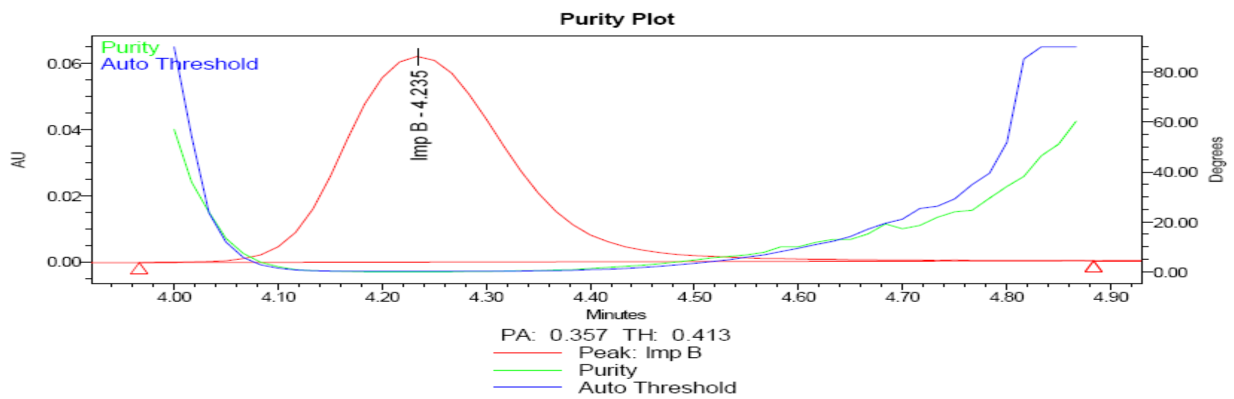

Figure 8: Peak purity of Impurity B

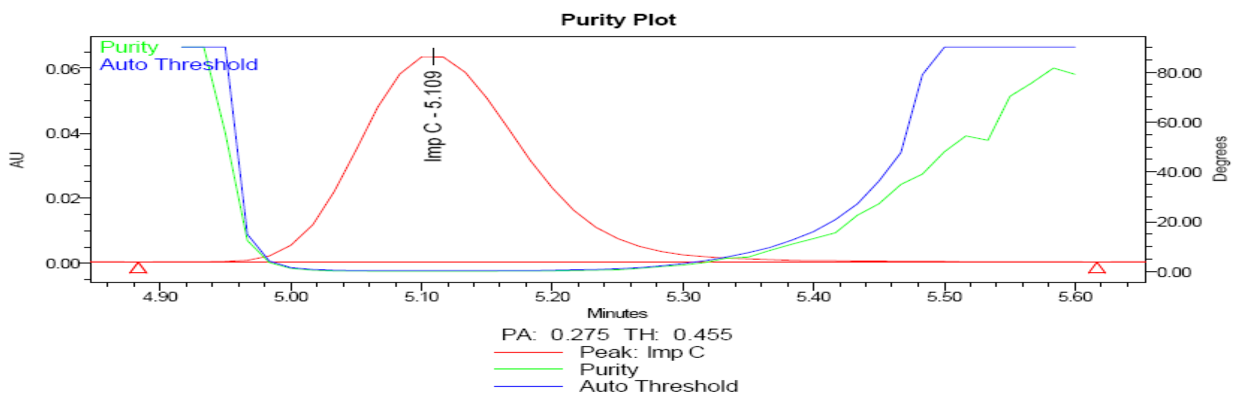

Figure 9: Peak purity of Impurity C

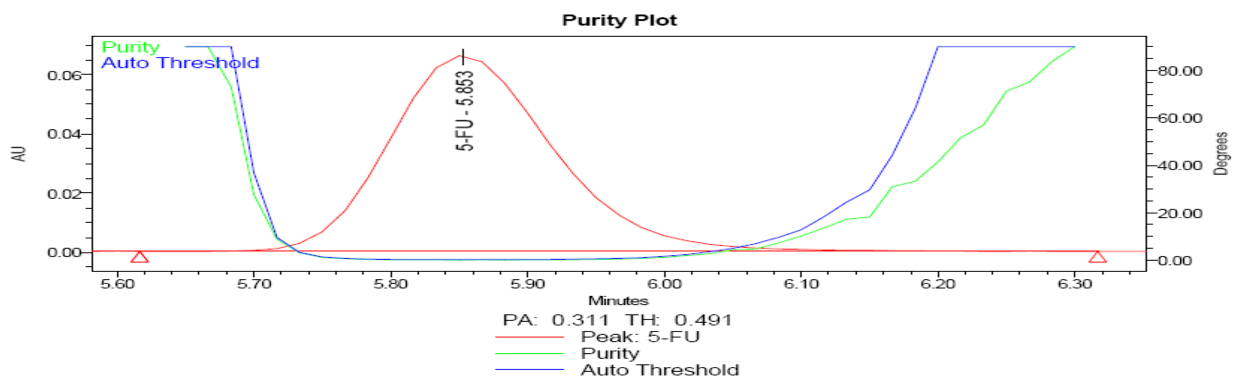

Figure 10: Peak purity of 5-Fluorouracil

licate injections of 5-Flurouracil, spiked with all impurities. The system deemed to be suitable as tailing factor ≤1.5, and theoretical plate > 8000, and resolution between closely eluting impurity >1.5 (figure 5).

ration containing 5-FU. The %RSD of 5-FU and impurity A, B, C in six sample preparation was found to be less than 2.0%.

Table 2: Peak purity of 5-FU and related impurities in stressed condition.

Stress condition	% Degradation	Purity angle	Purity threshold	Purity flag
Acid degradation	Nil	0.289	0.456	No
Base degradation	Nil	0.112	0.398	No
Peroxide degradation	Nil	0.312	0.498	No
Photo light degradation	Nil	0.344	0.512	No
UV light degradation	Nil	0.422	0.492	No
Heat degradation	Nil	0.379	0.428	No
Control	Nil	0.357	0.463	No

Table 3: System suitability parameters

S.No	Retention Time	USP Tailing	USP Resolution	Theoritical plates	Purity angle	Purity Threshold
5 FU	6.62	1.17	4.19	13881	0.311	0.491
Impurity A	3.67	1.27	NA	8615	0.150	0.272
Impurity B	4.60	1.25	5.52	11168	0.357	0.413
Impurity C	5.73	1.22	5.93	13763	0.275	0.455

Table 4: Linearity of 5-Fluorouracil from LOQ level to 500% of target sample concentration

	Linearity of 5-Fluorouracil	
Concentration in %	**Concentration in µg/ml**	**Area response**
LOQ	0.01006	1351
0.04%	0.04024	3214
0.08%	0.08048	5735
0.1%	0.1	6515
0.2%	0.2012	13674
0.5%	0.5	35258
1%	1	70159
2%	2	151499
10%	10	686635
100%	100	6348752
500%	500	29592036

3.4.3. Linearity, LOD & LOQ

The linearity solutions were prepared in mobile phase (diluent). Analyte solution has shown linear response for concentration levels ranging from 0.01µg/ml to 500µg/ml. The correlation co-efficient value was found to be 0.999. The relative response factor (RRF) was determined by slope method. LOD and LOQ of all impurities were calculated by STEYX method. The % rsd of LOD and LOQ samples were well within the limits of 33.3% and 10% respectively (table 4 & 5). The linearity plot is shown in (figure 11).

3.4.5. Precision

The method was found to be precise with six sample preparations for the quantification of 5-FU and its impurities. Impurity solution spiked to the sample preparation containing 5-FU.

3.4.6. Accuracy

The recovery of impurities-A, B, C and 5-FU were determined by spiking each impurity at six different levels starting from LOQ to 150% of the label claim of the drug product. The recovery range for all impurities was found to be between 87-108% with RSD between 0.42% and 4.59% (table 7).

3.4.7. Solution stability

The solution stability of the standard and impurities prepared in mobile phase was studied for 5 days at bench top. The solution under study was compared with freshly prepared standard solution, the samples were found to be stable for period of more than 72 hours.

Table 5: Linearity, LOD, LOQ, RRF, RRT of 5-Fluorouracil, impurity A, impurity B and impurity C

	Imp A		Imp B		Imp C		5 FU	
Desired Concentration	Conc in µg/ml	Area response	Conc in µg/ml	Area response	Conc in µg/ml	Area response	Conc in µg/ml	Area response
0.01	0.014	1896	0.012	1150	0.0105	831	0.010	1351
0.04	0.047	7092	0.048	2170	0.042	3312	0.040	3214
0.08	0.094	14227	0.096	5135	0.084	6603	0.080	5735
0.2	0.286	34188	0.240	13437	0.210	17276	0.201	13674
0.4	0.472	65491	0.480	27055	0.420	35237	0.402	26053
0.6	0.618	100833	0.618	41354	0.610	55323	0.603	39522
0.8	0.811	133244	0.803	54558	0.810	72341	0.804	50493
Slope		11607		68573		91416		62953
R^2		0.999		0.999		0.999		0.999
LOD(µg/ml)	0.004		0.004		0.01		0.004	
LOQ (µg/ml)	0.014		0.012		0.02		0.01	
RRF	1.9		0.9		1.4		NA	
RRT	0.55		0.69		0.89		NA	

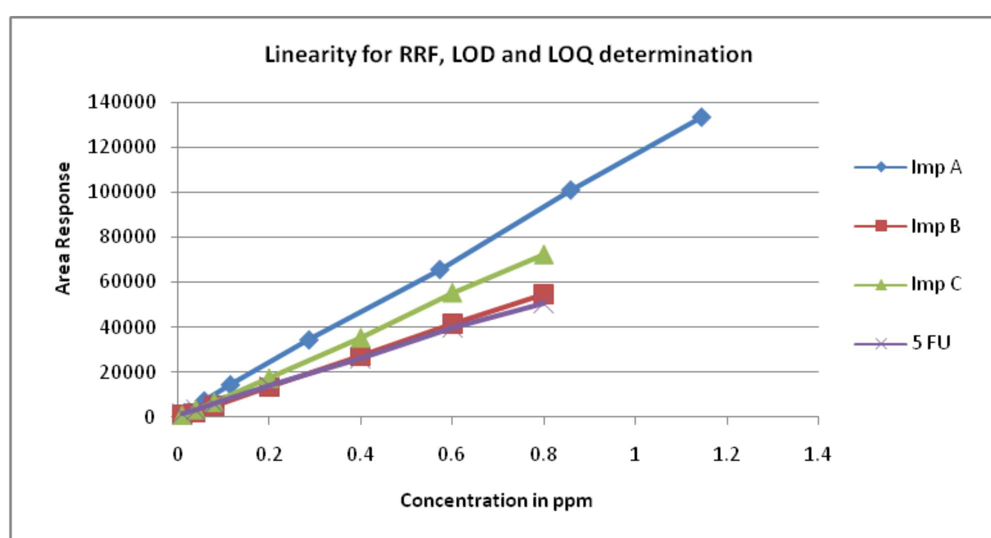

Figure 11: Linearity of 5-Fluorouracil, Impurity-A,B &C

Table 6: Assay value of six preparations of drug samples

Sample No.	% Assay	Total impurities
Sample 1	99.5	0.02
Sample 2	99.8	0.02
Sample 3	100.2	0.022
Sample 4	100.8	0.021
Sample 5	100.4	0.02
Sample 6	101.2	0.021
Mean	100.316	0.021
SD	0.6274	0.00081
% RSD	0.03	3.951

3.4.8. Robustness

The robustness was investigated by varying the conditions w.r.t. change in flow rate, pH and column temperature. The study was conducted at different flow rates of 0.8ml/min, and 1.2ml/min. The mobile phase pH was modified to 5.5, 6.5 and column temperature was adjusted to 22.5°C, and 27.5°C to study the effect of pH and column temperature respectively. Standard solution with six replicate injections, resolution solution containing the mixture of all the impurities and sample solution were injected. The method was found to be robust with respect to flow rate, pH and column temperature with out any changes in system suitability parameters such as tailing, resolution and theoritical plate (table 8).

CONCLUSION

The chemistry of column employed in present method allows working with 100% aqueous mobile phase showing excellent chromatographic features with respect to 5-FU and related substances. The method also provides selective quantification of 5-FU and impurities without interference from blank and placebo, thereby

Table 7: Recovery data of 5-FU and all impurities from LOQ to 150%

	Impurity A		Impurity B		Impurity C		5-FU	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
LOQ	92.69	2.13	87.01	1.53	94.13	2.16	94.54	2.78
50%	103.62	0.42	96.57	3.94	112.31	1.61	115.34	2.30
75%	97.71	1.17	90.77	1.14	97.69	0.66	98.53	1.43
100%	101.37	2.14	108.90	4.91	100.01	1.74	98.08	2.35
125%	112.79	4.59	105.83	4.12	108.84	2.37	108.37	0.32
150%	100.56	4.10	98.51	2.53	107.44	3.75	110.67	2.90

Table 8: Robustness

Parameters	Flow rate			pH			Column Temperature		
Changes in parameter	0.8 ml/min	1.0 ml/min	1.2 ml/min	5.5	6.0	6.5	22.5°C	25°C	27.5°C
% RSD of Standard injections	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Resolution	5.3	4.4	4.9	4.3	4.4	2.6	3.6	4.4	2.6
The tailing factor for 5-FU peak	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0	NMT 2.0
RRT of Imp A	0.83	0.55	0.84	0.58	0.55	0.61	0.55	0.55	0.58
RRT of Imp B	0.68	0.69	0.68	0.71	0.69	0.73	0.69	0.69	0.73
RRT of Imp C	0.83	0.89	0.84	0.87	0.89	0.92	0.86	0.89	0.88

affirming stability- indicating nature of method. The proposed method is highly sensitive, reproducible, specific and rapid. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method was robust in the separation and quantification of FU and related substances. This method can be used for the routine analysis of production samples. The information presented herein could be very useful for quality monitoring of bulk samples and as well employed to check the quality during stability studies.

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