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Research Article

ANALYTICAL METHOD VALIDATION FOR THE IDENTIFICATION AND DETERMINATION OF LIMIT OF UREA BY HPLC IN 2, 4-DIHYDROXY-5-FLUOROPYRIMIDINE INJECTION

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ABSTRACT

HPLC method is developed for the estimation of Urea in 2,4-dihydroxy-5-fluoropyrimidine bulk drug and its formulation i.e2,4-dihydroxy-5-fluoropyrimidine Injection by using HPLC system with auto sampler and UV/PDA detector(Column: Atlantis Hilic Silica Column, 250mm x4.6mm, 5 µm, Flow rate: 1.0 mL/min, Column temperature:25°C.Injection Volume: 10 µl, Run time: 25 Minutes Detection wave length: 200 nm),all validation parameters including specificity (interference, forced degradation), Precision (system, method, intermediate),linearity, accuracy, range, robustness studied. Forced degradation (acid, base,peroxide, water, thermal, humidity, photo stability effect studied for 2,4-dihydroxy-5-fluoropyrimidine in bulk drug & its Injection formulation.

Keywords: 2, 4-dihydroxy-5-fluoropyrimidine, Urea limit, Validation, HPLC.

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INTRODUCTION

2,4-dihydroxy-5-fluoropyrimidine a medication which is used in the treatment of cancer. It causes irreversible inhibition of thymidylate synthase. This drug is cell-cycle specific. They attack cells at very specific phases in the cycle [1-3].

Urea is most abundantly seen impurity in bulk drug & its injection formulation,

To identify, assay the limit of urea, no method was reported in literature. HPLC method was developed for the estimation urea ,Validated for all the parameters Developed method can be very effective in bulk drug industry &even in formulation sites for knowing the limit of urea ,as urea accumulation in the body leads to major side effects such as Gout, severity this disease can be further enhanced in presence of cancer [4]. Now a day's adopted synthetic pathways for 2,4-dihydroxy-5-fluoropyrimidine cannot avoid the presence of urea impurity ,So development of method for limiting the urea is mandatory for bulk drug & its formulation industry in the pharmaceutical quality control &quality assurance departments [5-7].

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MATERIALS & METHODS

2, 4-dihydroxy-5-fluoropyrimidine From Ther Dose Parma Pvt. Ltd, Hyderabad, Telangana, India And Urea, Ammonium acetate Sodium

hydroxide, Acetonitrile and potassium dihydrogen phosphate, Hydrochloric acid and hydrogen peroxide of analytical grade (A) were procured from S. D. Fine Chem. Ltd., Mumbai, INDIA.

Description of Analytical Method
Chromatographic parameters:
HPLC system: HPLC system with auto sampler and UV/PDA detector
Column: Atlantis Hilic Silica HPLC
Column, 250mm x4.6mm, 5 µm
Flow rate: 1.0 mL/min
Column temperature: 25°C
Injection Volume: 10 µL
Run time: 25 Minutes
Detection wave length: 200 nm

Preparation of buffer:
Prepare 20mM of Ammonium acetate in water.

Preparation of Mobile phase
Prepare a solution of Acetonitrile, buffer in the ratio 9:1.

Preparation of Reference solution
Weigh and transfer 25 mg ofUrea CRS into a 25 mL volumetric flask, dissolve and dilute to the volume with diluents.

Preparation of Sample solution (in duplicate)
Transfer 5 mL of Fluorouracil injection (50mg/mL) into a 10 mL volumetric flask, dissolve and dilute to the volume with diluents.

Procedure
Separately inject 10 µL of blank (diluents), reference solution in six replicates and sample solution into the chromatographic system. Record the chromatograms and measure the area response for urea peak.

System suitability criteria:
% RSD for peak area of Urea from six replicate injections of Reference solution should be not more than 5.0.

Chromatogram processing and disregard of peaks: Exclude any peaks that correspond to diluents responses.
Calculation: Calculate the percentage of the Urea in Fluorouracil injection according to the below equation.

Percentage Urea= [(rU x Cs) x100]/[rS xCu]
Where,
rU: Area of Urea in sample solution
rS : Average area of Urea in Reference standard solution
Cs: Concentration (mg/mL) of the Reference standard solution
Cu: Concentration (mg/mL) of the Test Solution

Reporting:
Report the Urea mean value as percentage to the two decimal points.

Acceptance criteria
Urea: NMT 4.0 %

Validation Results
System Suitability: As per methodology, injected blank, reference solution for six times into HPLC system**.**

Specificity:
Interference Study
As per methodology, injected blank, placebo solution once each and reference solution, sample solution and spiked sample solution and checked the peak interference of blank, placebo at the retention time of Urea. Prepared and injected Urea at specification level individually and checked the interference at retention time of Urea.

Forced Degradation study
Applied the stress conditions to the samples and then injected into HPLC System [8].

Acceptance Criteria:
% RSD for peak area of Urea from six replicate injections of Reference solution should be not more than 5.0
The blank and placebo should not show any peak at the retention time of Urea.
The peak purity should pass for Urea and the net degradation should be at least 5 to 20 % and the mass balance should be 90 % to 110 % at any of the condition. If the degradation is not achieved at any of the condition, report the minimal values.

Precision:
System Precision:
As per methodology, injected blank and reference solution six times into HPLC system.

Method Precision
Analyzed six test preparations of Fluorouracil injection 50 mg/mL spiked with urea at specification level as per the methodology and

determined the % RSD of six sample preparations of Fluorouracil.

Intermediate Precision

Determined the Intermediate precision by preparing six test preparations of Fluorouracil injection 50 mg/mL spiking urea at specification level as per the methodology and analyzed as per the test method by different analyst on different day by using different system with different column. Intermediate precision which was performed as a co-validation (inter laboratory variation) and considered for method transfer activity.

Establishment of Limit of Detection and Limit of Quantification:

As per methodology, injected blank, reference solution for six times and then injected LOD & LOQ Solutions into HPLC.

Precision at LOQ

Prepared and Injected the LOQ solution six times and reported the % RSD peak area of Urea.

Accuracy at LOQ Level

Prepared and injected the accuracy solutions at LOQ level, calculated the % recovery for Urea.

Linearity

Linearity for Urea was determined in the concentration range from LOQ to 150 % levels of specification level.

Accuracy

As per methodology, prepared sample solution by spiking Urea on Fluorouracil Injection 50 mg/mL at 50%, 100% and 150% and demonstrated the accuracy on sample into HPLC. Calculated the system suitability parameters and % mean recovery.

Acceptance criteria

% RSD for peak area of Urea from six replicate injections of Reference solution should be not more than 5.0.

Individual and mean % recovery value at 50%, 100% and 150% should be in between 90 to 110.

% RSD for the recovery at LOQ, 100 and 150% level should be not more than 10.0.

Range: From the results of Method Precision, Linearity and Accuracy it was concluded that the range of the Analytical method was established from LOQ to 150 % of target concentration

STABILITYOFANALYTICAL SOLUTION:

Stability study of standard solution and sample preparation were performed at room temperature and 2-8 °C conditions.

Robustness:

Effect of Variation in Flow rate:

System suitability preparations were analyzed as per the methodology at low column flow (0.9 mL/min) and high column flow (1.1 mL/min) variation in flow rate.

Effect of Variation in mobile phase composition:

System suitability preparations were analyzed as per the methodology at low buffer (890:110) and high buffer (910:90) variation in mobile phase composition.

Acceptance criteria

% RSD for peak area of Urea from six replicate injections of Reference solution should be not more than 5.0.

Effect of Variation in Column Oven Temperature\System suitability preparations were analyzed as per the methodology at high column Oven temperature (30°C) variation in column Oven temperature.

Table 1. Forced Degradation study: System suitability

Parameter	% RSD
Result	0.6
Acceptance Criteria	NMT 5.0

Table 2. Interference from Degradation process in blank

Name of Condition	Stress Condition	Interference at RT of Fluorouracil (Yes/No)
Acid	1.0 mL of 0.1 N HCl for 60 min at 60°C	No
Base	1.0 mL of 0.1 N NaOH for 60 min at 60°C	No
Peroxide	1.0 mL of 30 % H_2O_2 for 60 min at 60°C	No
Thermal	105°C for 6 hours	No

Humidity	90 % RH for 5 days	No
Photo Stability	1.2 million lux hours for white light and /200Watts for square meter for UV light	No

Table 3. Interference from Degradation process in Placebo

Name of Condition	Stress Condition	Interference at RT of Fluorouracil (Yes/No)
Acid	1.0 mL of 0.1 N HCl for 60 min at 60°C	No
Base	1.0 mL of 0.1 N NaOH for 60 min at 60°C	No
Peroxide	1.0 mL of 30 % H_2O_2 for 60 min at 60°C	No
Thermal	105°C for 6 hours	No
Humidity	90 % RH for 5 days	No
Photo Stability	1.2 million lux hours for white light and /200Watts for square meter for UV light	No

Table 4. Complete Degradation Data

S.No	Type of Stress	Assay (%w/w)	Degradation (%w/w)	Purity 1Angle	Purity 1 Threshold	Peak Purity (Pass/Fail)
1	**Acid**	94.7	5.3	0.69	1.732	Pass
2	**Base**	94.1	5.9	0.63	1.809	Pass
3	**Peroxide**	96.4	3.6	1.77	2.020	Pass
4	**Thermal**	98.8	1.2	0.54	1.089	Pass
5	**Humidity**	97.2	2.8	0.46	1.183	Pass
6	**Photo stability**	99.2	0.8	0.69	1.643	Pass

Table 5.Method Precision: System suitability

Parameter	% RSD
Result	0.3
Acceptance Criteria	NMT 5.0

Table 6. Method precision Results

Sample	Urea content (%w/w)
01	3.9
02	4.0
03	4.0
04	3.9
05	4.0
06	4.0
Average	4.0
S.D	0.052
%RSD	1.3

Table 7. Intermediate Precision

Parameter	% RSD
Result	3.5
Acceptance Criteria	**NMT 5.0**

Table 8. Details of Analyst, Column, Day and Instrument

Parameter	Analyst-1	Analyst-2
Column ID Number	01173408013301	H-14-36
HPLC ID Number	VLS-DR/HPLC/15	HP1 (Agilent 1100)
Date of Analysis	2016.03.25	2015.04.10

Table 9. Intermediate precision Results

Sample	Urea content (%w/w)
01	4.3
02	4.1
03	4.2
04	4.3
05	4.0
06	3.8
Average	4.1
S.D	0.198
%RSD	4.8

Table 10. Results of Method precision and Intermediate Precision

Preparation	Analyst –I/Column-I/System-I
Sites	**Lab-I**
1	3.9
2	4.0
3	4.0
4	3.9
5	4.0
6	4.0
Avg	4.0
SD	0.052
%RSD	1.3
%RSD(12 Prep)	3.9

Table 11. Limit of detection and limit of quantification: System suitability

Parameter	%RSD
Result	0.3
Acceptance Criteria	NMT 5.0

Table 12. Limit of Detection and Limit of Quantification

Name	LOD (ppm)	LOQ (ppm)
Urea	40	80

Table 13. Precision at LOQ System suitability

Parameter	%RSD
Result	0.3
Acceptance Criteria	NMT 5.0

Table 14. Accuracy at LOQ Level (Urea)

Sample No.	Urea		
	Added (ppm)	Found (ppm)	% Recovery
1	3.201	3.113	97.3
2	3.201	3.148	98.3
3	3.201	3.208	100.2
4	3.201	3.180	99.3
5	3.201	3.189	99.6
6	3.201	3.182	99.4
Mean			99.0
Std.dev			1.0420
% RSD			1.1

Table 15. Linearity: System suitability

Parameter	% RSD
Result	0.2
Acceptance Criteria	NMT 5.0

Table 16. Linearity Results of Urea

Level (%w/w)	Urea Concentration (ppm)	Urea Peak Area
LOQ	80.029	76877
50	500.180	481265
75	750.270	710326
100	1000.360	943481
125	1250.450	1176475
150	1500.540	1402659
Correlation Coefficient	1.000	

Table 17. Accuracy of Fluorouracil

Sample No	Spike level	Added (ppm)	Found (ppm)	'%' Recovery	'%' Mean recovery	%RSD
1	50%	20.007	20.050	100.2	100.0	0.2
2	50%	20.007	20.054	100.2		
3	50%	20.007	20.042	100.2		
4	50%	20.007	20.105	100.5		
5	50%	20.007	20.095	100.4		
6	50%	20.007	19.985	99.9		
1	100%	40.014	39.541	98.8	99.0	0.1
2	100%	40.014	39.624	99.0		
3	100%	40.014	39.668	99.1		
1	150%	60.022	58.539	97.5	98.0	0.5
2	150%	60.022	58.991	98.3		
3	150%	60.022	59.121	98.5		
4	150%	60.022	59.182	98.6		
5	150%	60.022	59.384	98.9		
6	150%	60.022	59.379	98.9		

Table 18. Stability of analytical solution: System suitability

Parameter	% RSD
Initial	0.3
Day1	0.2
Day2	0.1
Acceptance Criteria	**NMT 5.0**

Table 19. Assay Standard solution stability results (RT and 2-8°C)

Parameter		Standard Similarity factor
Initial		-
Day-1	**Standard at 2-8°C**	0.97
	Standard at RT	0.98
Day-2	**Standard at 2-8°C**	0.97
	Standard at RT	0.97

Table 20. Assay Sample solution stability results (RT and 2-8°C)

Parameter		% Urea content	% Difference from Initial
Initial		4.06	-
Day-1	Sample at 2-8°C	4.02	0.04
	Sample at RT	4.01	0.50
Day-2	Sample at 2-8°C	4.03	0.03
	Sample at RT	4.02	0.04

Table 21. Effect of Variation in Flow rate System suitability

Parameter	% RSD
Low flow	0.3
High flow	0.3
Acceptance Criteria	NMT 5.0

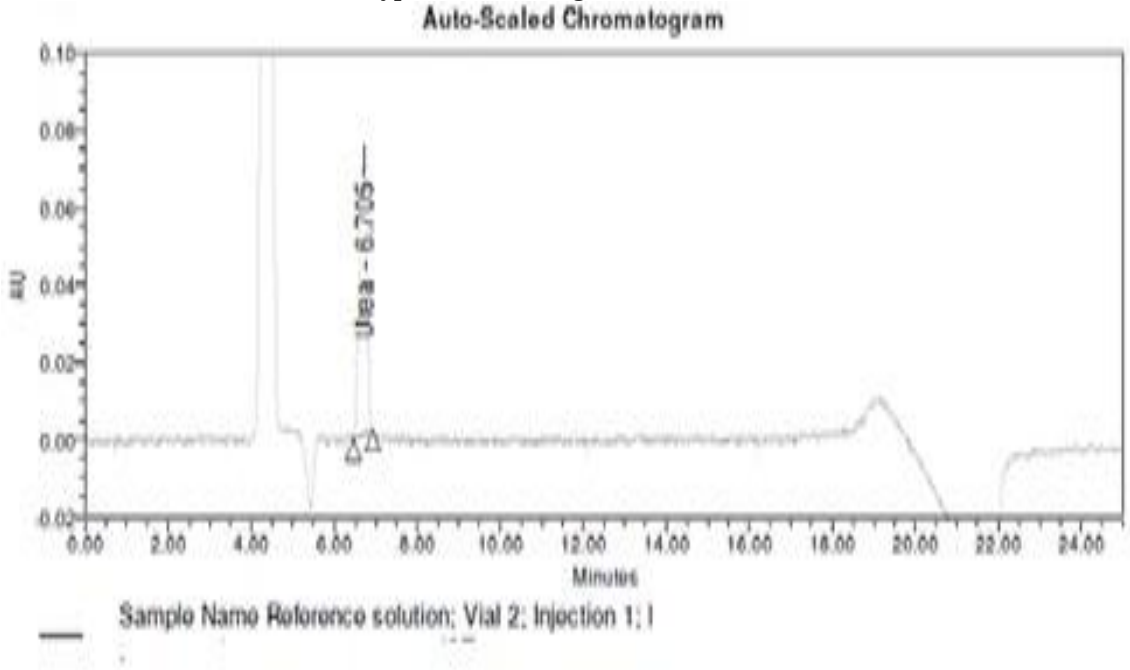
Table 22. Effect of Variation in mobile phase composition System suitability

Parameter	% RSD
Low organic	0.3
High organic	0.2
Acceptance Criteria	NMT 5.0

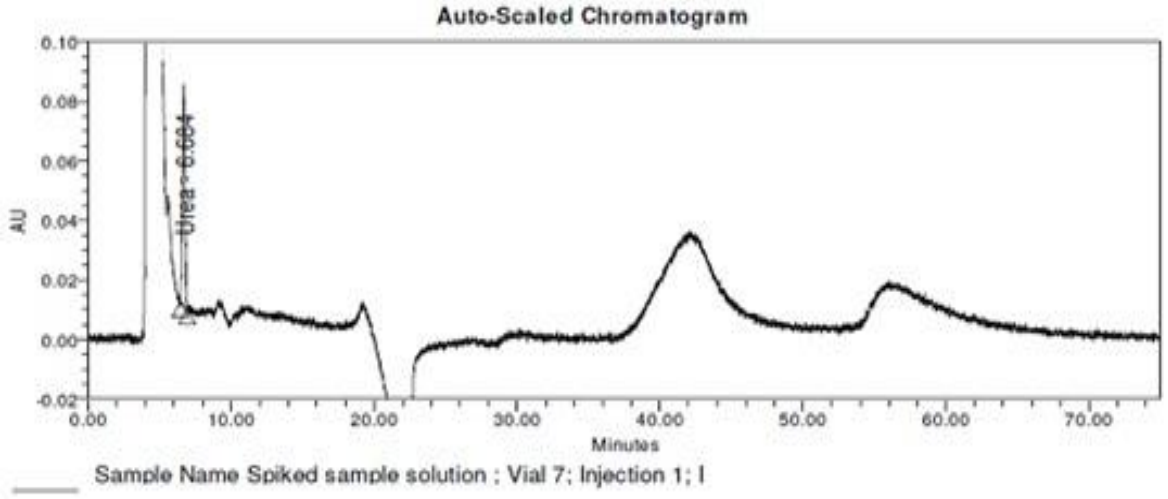
Table 23.Effect of Variation in Column Oven Temperature:

Parameter	% RSD
Low Temperature	0.4
High Temperature	0.5
Acceptance Criteria	NMT 5.0

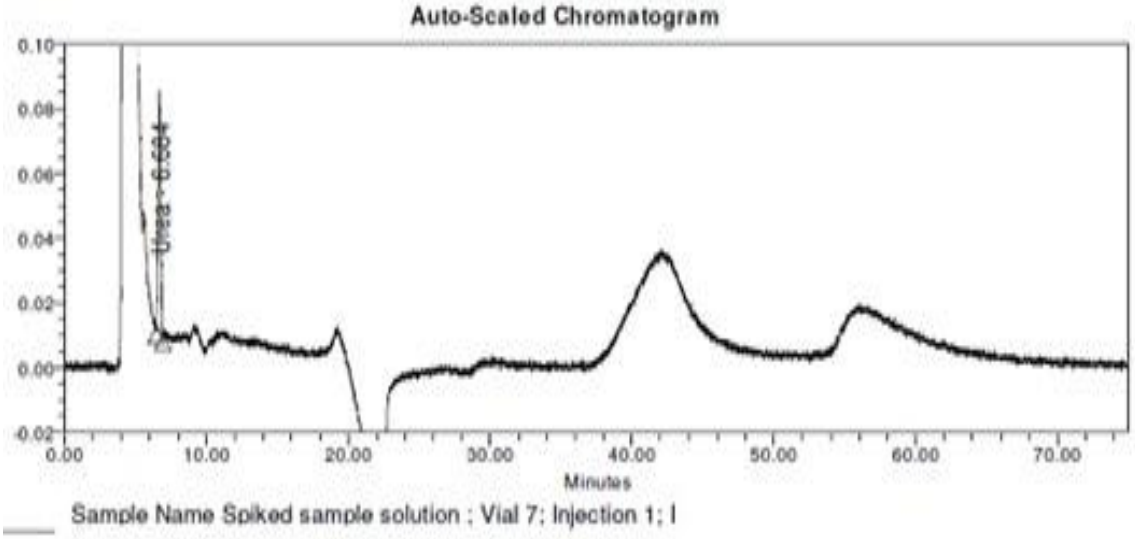
1. Typical Chromatogram of Standard



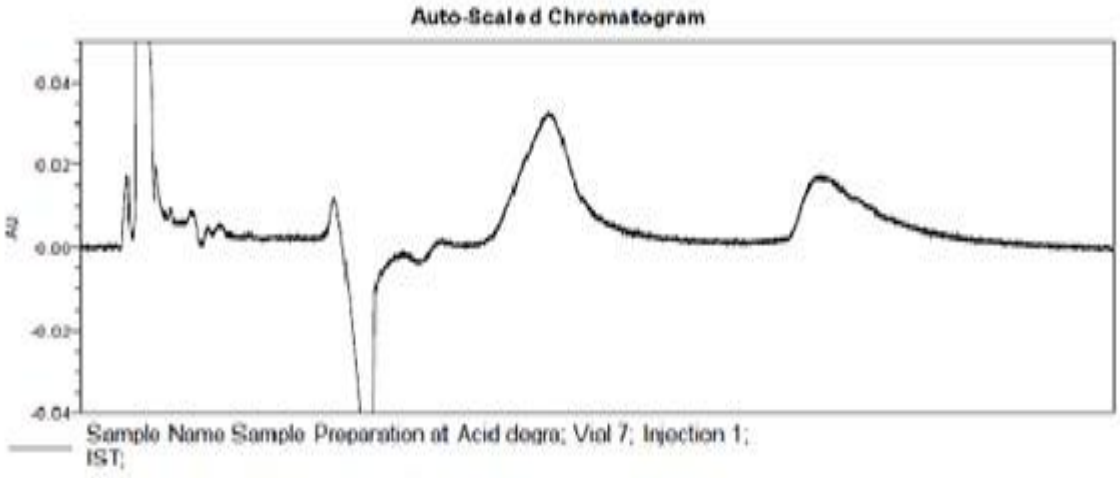
2.Typical Chromatogram of Sample



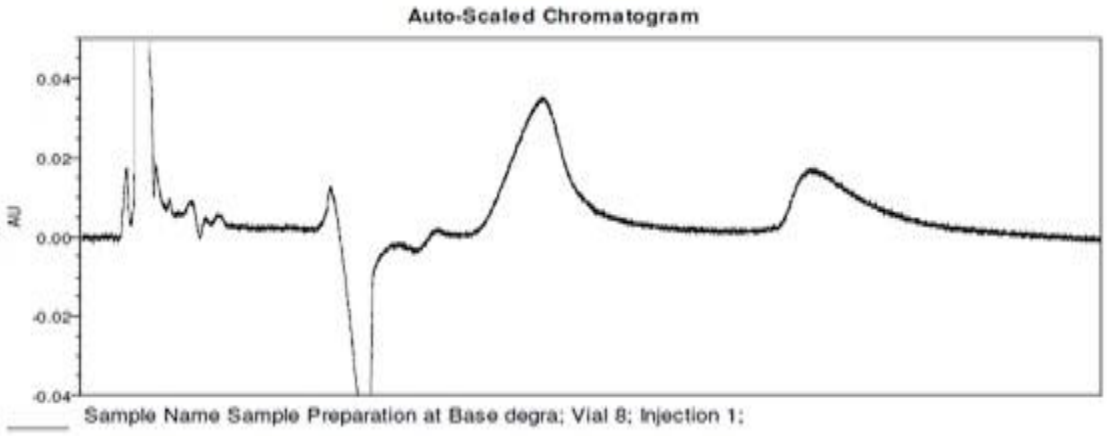
3.Typical Chromatogram of Spiked Sample



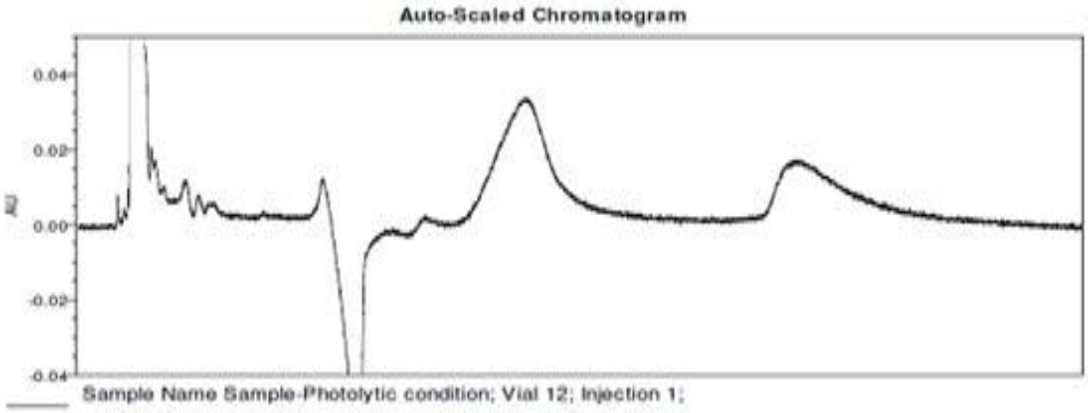
4. Typical chromatogram of Acid Stress Sample



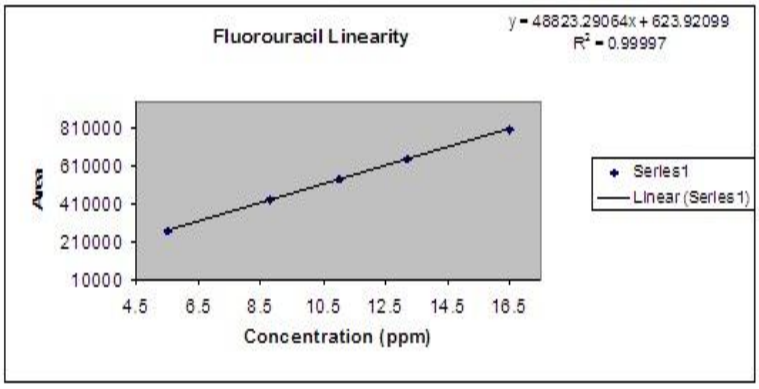
5. Typical Chromatogram of Base Stress Sample



6. Typical Chromatogram of Photolytic Stress Sample



7. Fluorouracil Linearity Graph



CONCLUSION

The present analytical method was validated as per defined protocol and it meets the specified acceptance criteria. Hence, it was concluded that the analytical method is specific, precise, linear, accurate, rugged and robust. The standard and sample solutions were stable up to two days at both room temperature and 2–8 °C. Hence, the present analytical method proved as stability indicating, and can be used for regular analysis and its intended purpose.

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REFERENCES

1. Karger, Barry L. HPLC: Early and Recent Perspectives. Journal of Chemical Education. 74, 1997, 45. Doi:10.1021/ed074p45.
2. Giddings, J. Calvin. Dynamics of Chromatography, Part I. Principles and Theory. Marcel Dekker, Inc., New York. 1965, p. 281.
3. ICH, Stability testing: Photo stability testing of new drug substances and products. Geneva: International Conference on Harmonization, IFPMA; 1996.
4. Bakshi M, Singh S. Development of validated stability-indicating assay methods-critical review. *J Pharma Biomed Anal*, 28, 2002, 1011–40.
5. Henry, Richard A. The Early Days of HPLC at DuPont". Chromatography Online. Avanstar Communications Inc, 2009.
6. Sinha VR, Kumar RV, and Bhinge JR. A Stability-Indicating RP-HPLC Assay Method for 5-Fluorouracil. *Indian Journal of Pharmaceutical Sciences*, 71(6), 2009, 630–637.
7. ICH, Stability testing of new drug substances and products. Geneva: International Conference on Harmonization, *IFPMA*, 2003.
8. Garret ER, Nestler HJ, Somodi A. Kinetics & mechanism of hydrolysis of 5-halouracils. *J Org Chem*, 33, 1968, 3460–8.

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