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Stability Indicating Analytical Method Development, Validation, Method Transfer and Impurity Profile (Related Substances) Of 2,4-Dihydroxy-5-Fluoropyrimidine By Liquid Chromatography

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ABSTRACT:- Stability indicating liquid chromatographic method was developed for related substances [(Pyrimidine-2,4,6 (1H,3H,5H)-trione ,Dihydropyrimidine-2,4,5(3H)-trione ,Uracil,5-Methoxy Uracil, 5-Chloropyrimidine-2,4(1H,3H)-dione)] of 2,4-dihydroxy-5-fluoro pyrimidine , validation was performed on higher presentation i.e. 5 g/100 mL by using Column dimensions of 250mm, 5µm (YMC Pack ODS AQ) with the Flow rate of 1.0 mL/min at Wavelength of 266 nm by Injecting Volume of 20 µL by maintain Run time of 30 minutes.

I. INTRODUCTION

2, 4-dihydroxy-5-fluoropyrimidine is an anti metabolite used in cancer chemotherapy, one of the drug of choice in the treatment of cancer, which acts by inhibition of the enzyme thymidylate synthase irreversibly.[2] . It attacks cells at very specific phases in the cycle.[1, 3, 7.] The impurities such as Pyrimidine-2,4,6 (1H,3H,5H)-trione ,Dihydropyrimidine-2,4,5(3H)-trione ,Uracil,5-Methoxy Uracil, 5-Chloropyrimidine-2,4(1H,3H)-dione were frequently found impurities in the synthesis, processing of 2,4-dihydroxy-5-fluoropyrimidine in bulk drug &formulation sites ,for identifying such impurities & assay the limit of above five impurities , no HPLC method was reported in literature. Forced degradation studies were performed for the developed method ,hence developed method is stability indicating , can be very effective in bulk drug premises &even in formulation sites for knowing the limit of impurities

II. MATERIALS &METHODS

Details of Chemicals: Fluorouracil Standard(Batch IOG371.USP Grade),Fluorouracil 50mg/ml(Batch IFU-319(B),Ingénues),Mono basic Potassium Phosphate (Batch QF4Q641420,Merck), Acetonitrile (Batch :IA51F65025,Merck)

Instruments (Columns, serial no.):
HPLC: VLS-DR/HPLC/05
VLS-DR/HPLC/12
VLS-DR/HPLC/16
VLS-DR/HPLC/17

Analytical Balance:VLS-DR/BAL/01
pH Meter: VLS-DR/PHM/01
Description of Analytical Method:
Chromatographic Parameters:
Column: 250mm, 5µm (YMC Pack ODS AQ or Phenomenex Luna C18 (2) 100A or equivalent to L1)
Flow rate: 1.0 mL/min
Wavelength: 266 nm
Injection Volume: 20 µL
Column oven Temperature: Ambient
Run time : 30 minutes

Preparation of Mobile Phase

Weigh about 6.8 g of Monobasic Potassium phosphate and transfer into 1000 ml of water, dissolve and adjust the pH of this solution to 5.7 with 5M Potassium hydroxide.

Fluorouracil Standard Stock solution

Accurately weigh and transfer 5 mg of Fluorouracil standard into a 25 mL volumetric flask, dissolve and dilute to volume with diluent. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluent.

Fluorouracil Related Compound A Stock solution (Stock-A)

Weigh and transfer 5 mg of Fluorouracil Impurity-A standard into a 25 ml volumetric flask, dissolve and dilute to volume with diluent. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluent

Fluorouracil Related Compound B Stock solution (Stock-B) Weigh and transfer 5 mg of Fluorouracil Impurity-B standard into a 25 ml volumetric flask dissolve and dilute to volume with diluent. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluents

Fluorouracil Related Compound C Stock solution (Stock-C)

Weigh and transfer 5 mg of Uracil (Impurity-C) standard into a 25 ml volumetric flask, dissolve and dilute to volume with diluents. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluents

Fluorouracil Related Compound D Stock solution (Stock-D)

Weigh and transfer 5 mg of Fluorouracil Impurity-D standard into a 25 ml volumetric flask, dissolve and dilute to volume with diluents. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluent

Fluorouracil Related Compound E Stock solution (Stock-E)

Weigh and transfer 5 mg of Fluorouracil Impurity-E standard into a 25 ml volumetric flask, dissolve and dilute to volume with diluent. Transfer 1 mL of this solution into a 100 mL volumetric flask and dilute to volume with diluent

Preparation of Standard solution

Transfer each 1.0 mL of Fluorouracil Standard Stock solution**,** Stock A, B, C, D, E into a 10 mL volumetric flask and dilute to volume with diluent and mix well.

Preparation of Test solution:

Transfer 1 mL of Fluorouracil injection (50mg/mL) into a 50 mL volumetric flask, dissolve and dilute to the volume with diluent. Transfer 1.0 mL of the above solution into a 10 mL volumetric flask and dilute to volume with diluent and mixed well.

III: VALIDATION

System Suitability

Injected blank and standard solution for six times into the HPLC system.Specificity

Interference Study:

As per methodology, injected blank, placebo solution once each and standardSolution, sample solution and spiked solution and checked the peak interference of blank, placebo and impurities at the retention time of Fluorouracil and its Impurities. Prepared and injected each impurity at 1% level individually and checked the interference at each impurity retention time.

Precision:

System Precision:

As per methodology, injected blank and standard solution six times and check standard once into HPLC system.

Method precision:

Analyzed six test preparations of Fluorouracil injection 50 mg/mL as per the methodology and determined the % RSD of any individual impurity and total impurities from six sample preparations of Fluorouracil.**Intermediate Precision: Determined** the Intermediate precision by preparing six test preparations of Fluorouracil injection 50 mg/mL as per the methodology and analyzed as per the test method by different analyst on different analyst on different day by using different system with different column. Here intermediate

precision study was carried out at the receiving site. Intermediate precision which was performed as a co-validation (inter laboratory variation) and considered for method transfer activity.

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.LinearityLinearity for Fluorouracil was determined in the concentration range from 50 to 150 % levels of test concentration levels.

Accuracy:

As per methodology, prepared 50%, 100% and 150% sample solutions of Fluorouracil working concentration and demonstrated the accuracy on sample into HPLC. Calculated the system suitability parameters and % mean recovery.

Range:

From the results of Method Precision, Linearity and Accuracy it was concluded that the range of the Analytical method was established from 50 to 150 % of target concentration.

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.

Acceptance criteria

The resolution between Fluorouracil and Uracil (Impurity-C) should be not less than 2.0 in the standard solution.The % RSD of the area of Fluorouracil peak from six replicate injections of standard solution should be not more than 5.0.

Effect of Variation in pH

System suitability preparations were analyzed were analyzed as per the methodology at low pH (5.6) and high pH (5.8) variation in buffer.

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.

IV. RESULTS &DISCUSSION:

Table.1: Relative Retention Time of Impurities

Name	**Relative Retention Time**
Fluorouracil Related compound A, Pyrimidine-2,4,6(1H,3H,5H)-trione.	0.5
5-Fluorouracil Related compound B, Dihydropyrimidine-2,4,5(3H)-trione	0.7
Uracil	0.9
Fluorouracil	1.0
5-Methoxy Uracil	1.6
Fluorouracil Related compound E, 5-Chloropyrimidine-2,4(1H,3H)-dione	1.9

Table.2: Placebo Interference Data

S.No.	**RT of Fluorouracil impurity A**	**RT of Fluorouracil impurity B**	**RT of Fluorouracil impurity C**	**RT of Fluorouracil impurity D**	**RT of Fluorouracil impurity E**
Interference found (Yes/No)					
1	No	No	No	No	No

Table.3: Impurities Interference Data

S.No	Name	Interference Due to other Impurities(Yes/No)
1	Fluorouracil impurity A	No
2	Fluorouracil impurity B	No
3	Fluorouracil impurity C	No
4	Fluorouracil impurity D	No
5	Fluorouracil impurity E	No

Table.4: **Interference from Degradation process in blank**

Name of Condition	Stress Condition	Interference at RT of Fluorouracil (Yes/No)
Acid	1.0 mL of 5 M HCl for 180 min at 60°C	No
Base	1.0 mL of 5 M NaOH for 180 min at 60°C	No
Peroxide	1.0 mL of 30 % H_2O_2 for 5 min at 60°C	No
Water	1.0 mL of Water for 60 min at 90°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 5: **Complete Degradation Data**

S.No	Type of Stress	Assay (%w/w)	Purity 1Angle	Purity 1 Threshold	Peak Purity (Pass/Fail)
1	**Acid**	95.2	0.59	1.645	Pass
2	**Base**	93.9	0.64	1.309	Pass
3	**Peroxide**	97.9	1.02	2.456	Pass
4	**Thermal**	99.0	0.45	1.465	Pass
5	**Humidity**	96.4	0.39	1.239	Pass
6	**Photo stability**	98.9	0.73	1.875	Pass

Table 6: Method precision Results

Sample	Any Individual impurity (%w/w)	Total impurities (%w/w)
01	0.0096	0.0249
02	0.0097	0.0260
03	0.0098	0.0265
04	0.0093	0.0264
05	0.0099	0.0252
06	0.0093	0.0243
Average	0.0096	0.0256
S.D	0.0003	0.0009
%RSD	2.6	3.5

Table.7: Limit of Detection and Limit of Quantification

Name	LOD (ppm)	LOQ (ppm)
Fluorouracil	0.0006	0.0014

Table.8: Precision at LOQ

Preparation	Area
1	907
2	904
3	899
4	944
5	870

6	921
Average	908
STDEV	24.5173
% RSD	2.7

Table.9: Accuracy at LOQ Level of Fluorouracil

Sample No.	**Fluorouracil**		
	Added	**Found**	**% Recovery**
1	0.00141	0.00136	96.45
2	0.00141	0.00138	97.87
3	0.00141	0.00125	88.65
Mean			94.3
Std.dev			4.964
% RSD			5.3

Table.10: Linearity Results of Fluorouracil

Level (%w/w)	**Fluorouracil Concentration**	**Fluorouracil Peak Area**
LOQ	0.0015	978
50	0.0101	5761
80	0.0161	9420
100	0.0201	11919
120	0.0241	14313
150	0.0302	17956
Correlation Coefficient	0.9998	

Table11: Accuracy of Fluorouracil (Assay)

Sample No	**Spike level**	**Added (mg/mL)**	**Found (mg/mL)**	**'%' Recovery**	**'%' Mean recovery**	**%RSD**
1	50%	0.01005	0.01002	99.66	99.8	0.1
2	50%	0.01005	0.01004	99.87		
3	50%	0.01005	0.01004	99.84		
1	100%	0.02010	0.02014	100.19	100.1	0.2
2	100%	0.02010	0.02007	99.82		
3	100%	0.02010	0.02016	100.26		
1	150%	0.03015	0.03032	100.56	100.3	0.3
2	150%	0.03015	0.03025	100.33		
3	150%	0.03015	0.03017	100.04		

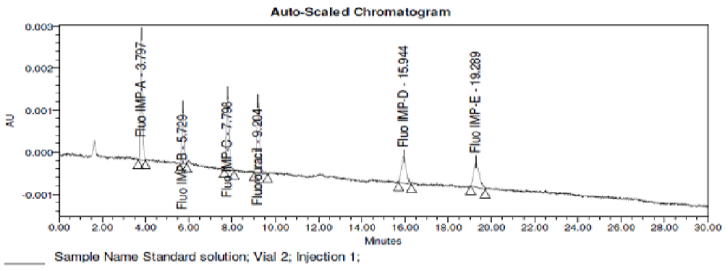
Table12: Assay Sample solution stability results (RT and 2-8°C)

Parameter		**Any individual impurity**	**% Difference from Initial**	**Total impurities**	**% Difference from Initial**
Initial		0.0096	-	0.0249	-
Day-1	**Sample at 2-8°C**	0.0094	0.0002	0.0264	0.0015
	Sample at RT	0.0072	0.0024	0.0253	0.0004
Day-2	**Sample at 2-8°C**	0.0123	0.003	0.0323	0.0074
	Sample at RT	0.0131	0.0035	0.0280	0.0031

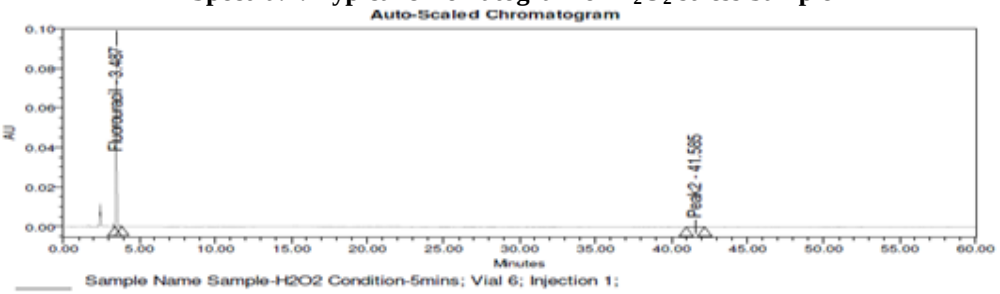
Table13: Robustness: Effect of Variation in Flow rate

Parameter	Resolution	% RSD
Low flow	4.6	1.1
High flow	5.1	0.3
Acceptance Criteria	**NLT 2.0**	**NMT 5.0**

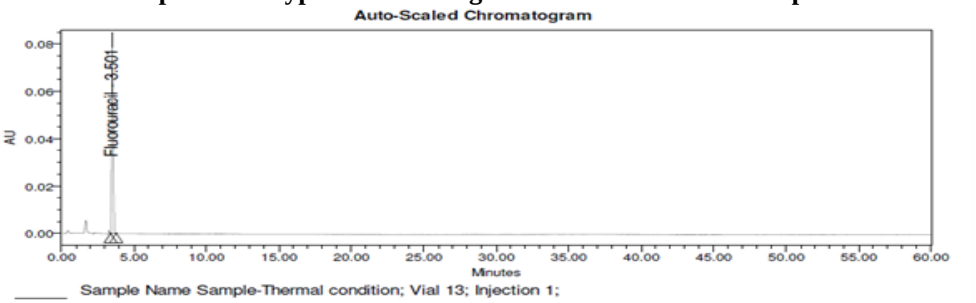
Spectra.1: Typical chromatogram of Standard



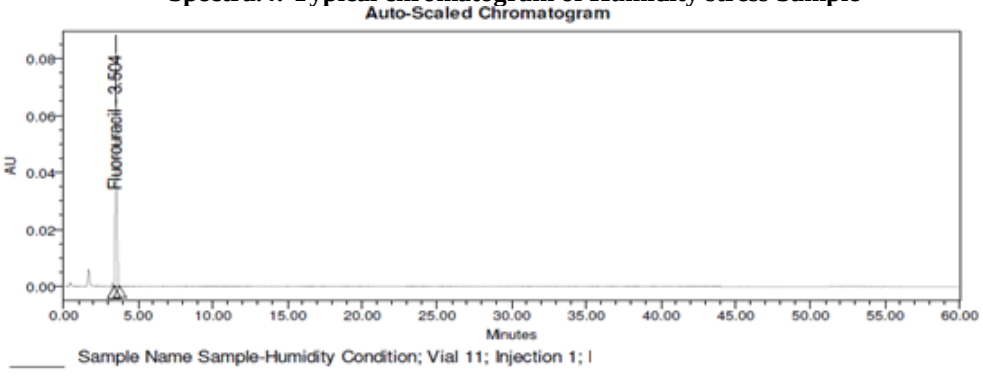
Spectra.2: Typical chromatogram of H_2O_2 stress Sample



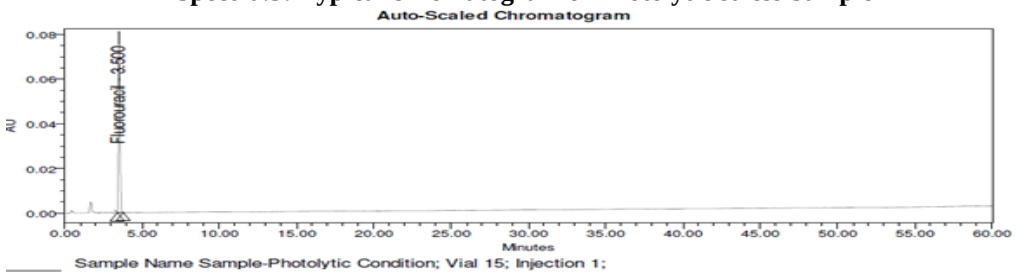
Spectra.3: Typical chromatogram of Thermal stress Sample



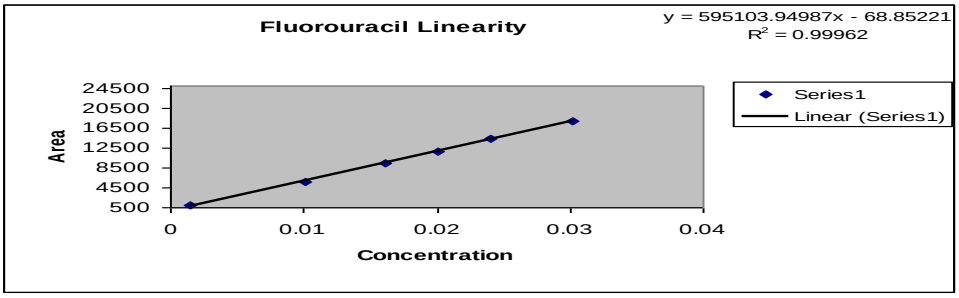
Spectra.4: Typical chromatogram of Humidity stress Sample



Spectra.5: Typical chromatogram of Photolytic stress Sample



GRAPH.1: Fluorouracil Linearity



V. CONCLUSION

Forced degradation studies of developed method for identifying the related substances of 2, 4 – dihydroxy -5-fluoropyrimidine were established. The present analytical method was validated for all the validation parameters and the developed analytical method meets the required acceptance criteria, the present analytical method proved to be stability indicating because the results were within the acceptance criteria both at transferring site and receiving site therefore the method transfer stands successful and can be used for regular analysis in pharmaceutical analysis &quality control departments for its intended purpose.

VI. ACKNOWLEDGEMENT

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