RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF TRIFLURIDINE AND TIRPIRACIL IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM

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ABSTRACT : A simple, rapid, precise, accurate and sensitive reverse phase liquid chromatographic method has been developed for the simultaneous determination of Trifluridine and Tipiracil in bulk and pharmaceutical dosage form dosage form. The chromatographic method was standardized using Waters ODS (C18) RP Column, 250 mm x 4.6 mm. 5µm i.d. column with UV detection at 251 nm and Phosphate Buffer (pH- 6.5): Acetonitrile with 65: 35% v/v ratios at a flow rate of 1.0 ml/ min. The Retention time of Trifluridine and Tipiracil in Optimized condition is 2.179 and 3.610. The proposed method was successfully applied to the simultaneous determination of Trifluridine and Tipiracil in bulk and pharmaceutical dosage form. The method was linear over the range of 0-60µg/ml for Trifluridine and 0-40µg/ml for Tipiracil. The recovery was in the range of 98% to 102%. The LOD was found to be 0.06 µg/ml and 0.08 µg/ml for Trifluridine and Tipiracil respectively. The LOQ was found to be 0.18 µg/ml and 0.24 µg/ml for Trifluridine and Tipiracil. The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Trifluridine and Tipiracil were stable only in acidic, basic and thermal stress conditions and photolytic stress conditions. Different analytical performance parameters such as precision, accuracy, limit of detection, limit of quantification and robustness were determined according to International Conference on Harmonization (ICH) guidelines.

Keywords: RP-HPLC, Trifluridine and Tipiracil, Accuracy, Precision, ICH Guidelines.

I.INTRODUCTION

Trifluridine/Tipiracil is the combination of an antineoplastic pyrimidine analogue (Trifluridine) with an inhibitor of its metabolism (Tipiracil) that is used in the therapy of refractory, metastatic colorectal cancer. Trifluridine/Tipiracil is associated with a low rate of transient serum enzyme elevations during therapy, but has not been implicated in cases of clinically apparent acute liver injury with jaundice. Trifluridine[1] is a fluorinated thymidine analog with potential antineoplastic activity. Trifluridine is incorporated into DNA and inhibits thymidylate synthase, resulting in inhibition of DNA synthesis, inhibition of protein synthesis, and apoptosis. This agent also exhibits antiviral activity. Trifluridine is a pyrimidine 2'-deoxyribonucleoside compound having 5-trifluoromethyluracil as the nucleobase. An antiviral drug used mainly in the treatment of primary keratoconjunctivitis and recurrent epithelial keratitis. It has a role as an antiviral drug, an antimetabolite, an EC 2.1.1.45 (thymidylate synthase) inhibitor and an antineoplastic agent. It is a nucleoside analogue, an organofluorine compound and a pyrimidine 2'-deoxyribonucleoside. The IUPAC Name of Trifluridine[2] is 1-[(2R, 4S, 5R)-4-hydroxy-5-(hydroxy methyl) oxolan-2-yl]-5-(trifluoromethyl) pyrimidine-2, 4-dione. The Chemical Structure of Trifluridine[3] is following

Fig.1. Chemical Structure of Trifluridine

Tipiracil[4] is a member of the class of pyrimidones that is uracil substituted by chloro and (2-iminopyrrolidin-1-yl) methyl groups at positions 5 and 6 respectively. Used (as the hydrochloride salt) in combination with Trifluridine, a nucleoside metabolic inhibitor, for treatment of advanced/relapsed unresectable colorectal cancer. It has a role as an antineoplastic agent and an EC 2.4.2.4 (thymidine phosphorylase) inhibitor. It is a pyrimidone, an organochlorine compound, a carboxamidine and a member of pyrrolidines. It derives from a uracil. It is a conjugate base of a Tipiracil[5] (1+). Trifluridine/Tipiracil is the combination of an antineoplastic pyrimidine analogue (Trifluridine) with an inhibitor of its metabolism (Tipiracil) that is used in the therapy of refractory, metastatic colorectal cancer. Trifluridine/Tipiracil is associated with a low rate of transient serum enzyme elevations during therapy, but has not been implicated in cases of clinically apparent acute liver injury with jaundice. Tipiracil is a thymidine phosphorylase inhibitor. It is used in combination with Trifluridine, in a ratio of 1:0.5, to form TAS-102. The main function of Tipiracil[6] in TAS-102 is to increase Trifluridine bioavailability by inhibiting its catabolism. TAS-102 is indicated for the treatment of metastatic colorectal cancer which has been previously treated with fluoropyrimidine-, oxaliplatin- and irinotecan-based chemotherapy, or with an anti-VEGF or anti-EGFR therapy. The IUPAC Name of Tipiracil is 5-chloro-6-[(2-iminopyrrolidin-1-yl) methyl]-1H-pyrimidine-2, 4-Dione. The Chemical Structure of Tipiracil is as follows

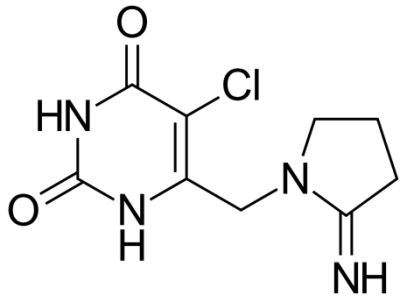

Fig.2. Chemical Structure of Tipiracil

II. EXPERIMENTAL

Table-1: List of Instrument used

S. No.	Instruments/Equipment/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator(Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table-2: List of Chemicals used

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai

2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Phosphate Buffer	99.9%	A.R.	Sd fine-Chem ltd; Mumbai

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Trifluridine and Tipiracil, so that the same wave number can be utilized in HPLC UV detector for estimating the Trifluridine and Tipiracil. The scanned UV spectrum[7] is attached in the following page.

Wavelength Detection (Or) Selection of Wavelength

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm.

Preparation of 0.02M Phosphate buffer solution (ph-6.5):

About 2.72168 grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. The pH[8] was adjusted to 6.5 with diluted Orthophosphoric acid.

Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Phosphate Buffer (pH- 6.5) and Acetonitrile at the volume of 1000ml. 650ml of Phosphate Buffer and 350ml of Acetonitrile were mixed well and degassed[9] in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration[10].

Preparation of Standard Solutions:

10 mg of Trifluridine and Tipiracil was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml mobile phase was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 10µg/ml and 10µg/ml of Trifluridine and Tipiracil respectively.

III. RESULTS AND DISCUSSION

Method Development

Selection of Wavelength

The detection wavelength[11] was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Trifluridine and Tipiracil was obtained and the isobestic point of Trifluridine and Tipiracil showed absorbance's maxima at 251 nm.

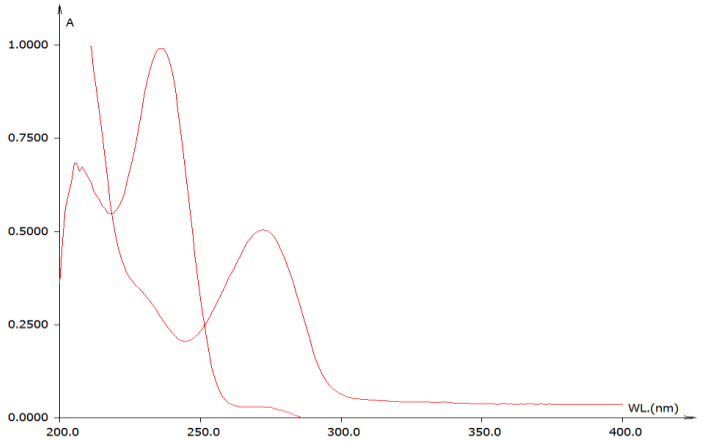

Fig.3.Overlay Spectrum for Trifluridine and Tipiracil

Optimized Chromatographic Method:

Table-3: Optimized Chromatographic Conditions

Mobile phase	Phosphate Buffer (pH- 6.5) : Acetonitrile = 65 : 35%v/v
Column	Waters ODS (C18) RP Column.
Column Temperature	Ambient
Detection Wavelength	251 nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

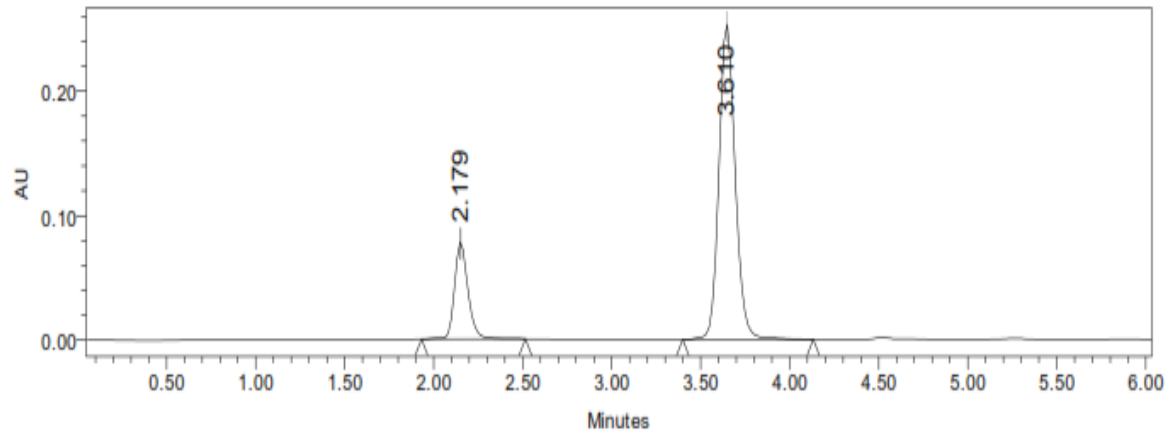

Fig.4. Chromatogram of Trifluridine and Tipiracil in Optimized Chromatographic Condition

Method Validation

System Suitability: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability[12-13] test parameters were established. The data are shown in Table-4.

Table-4: System suitability results for Trifluridine and Tipiracil (Flow rate)

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	3.87
2	Asymmetry	T ≤ 2	Trifluridine = 0.22 Tipiracil = 0.32
3	Theoretical plate	N > 2000	Trifluridine = 2956 Tipiracil= 3028

Linearity: To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase[14] to get a series of concentration ranging from 0-60µg/ml for Trifluridine and concentration ranging from 0-40µg/ml for Tipiracil. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[15] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of calibration graphs:

The resultant areas of linearity[16] peaks are plotted against Concentration.

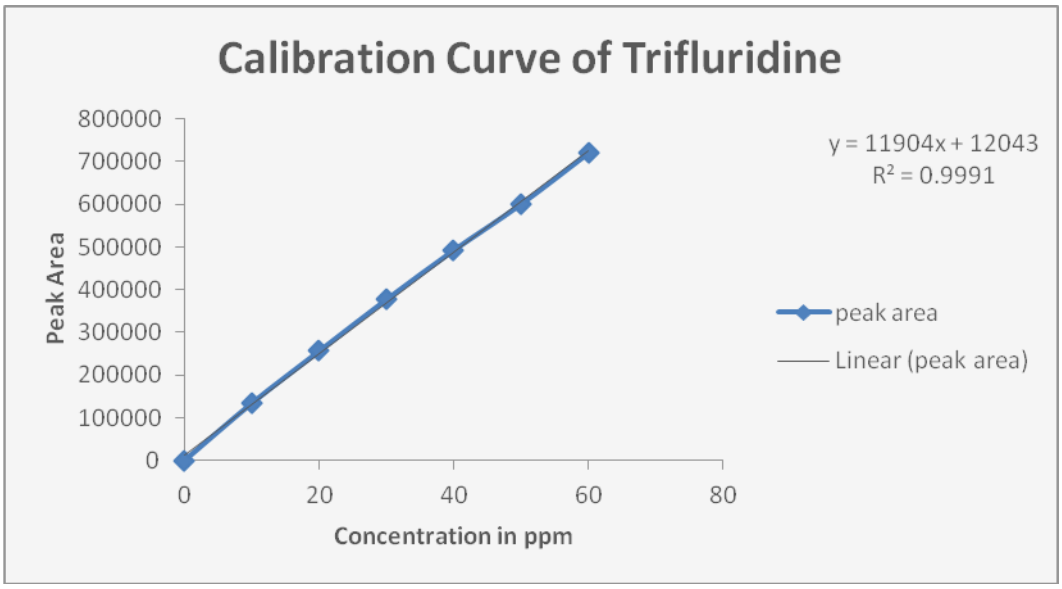

Fig.5. Standard curve for Trifluridine

Table-5: Linearity Readings for Trifluridine

CONC. (µg/ml)	AUC (n=6)
0	0
10	134528
20	256580
30	377574
40	493125
50	601256
60	721010

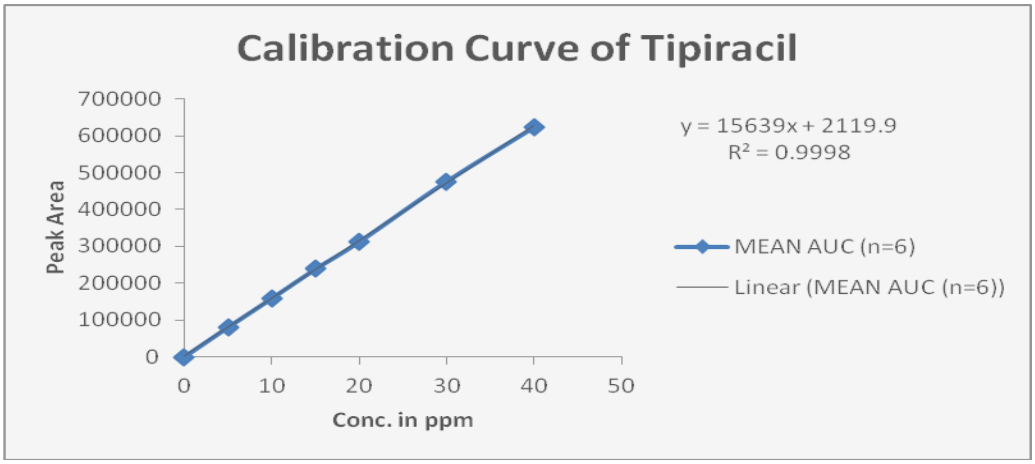

Fig.6. Standard curve for Tipiracil

Table-6: Linearity Readings for Tipiracil

CONC. (µg/ml)	MEAN AUC (n=6)
0	0
5	80586
10	158963
15	238722
20	312830
30	476594
40	623852

Observation: Linearity range[17] was found to be 0-60 µg/ml for Trifluridine. The correlation coefficient was found to be 0.999, the slope was found to be 11904 and intercept was found to be 12043 for Trifluridine. Linearity range was found to be 0-40 µg/ml for Tipiracil. The correlation coefficient[18] was found to be 0.999, the slope was found to be 15639 and intercept was found to be 2119 for Tipiracil.

Accuracy:

Table-7: Accuracy results of Trifluridine

Sample ID	Concentration (µg/ml)			% Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	40	39.947	487574	99.867	Mean= 100.4113% S.D. = 0.473694 % R.S.D.= 0.471754
S_2 : 80 %	40	40.255	491241	100.637	
S_3 : 80 %	40	40.292	491685	100.73	
S_4 : 100 %	50	49.705	603735	99.41	Mean= 100.6647% S.D. = 1.166369% R.S.D.= 1.158668
S_5 : 100 %	50	50.434	612421	100.868	
S_6 : 100 %	50	50.858	617459	101.716	
S_7 : 120 %	60	59.927	725421	99.878	Mean= 100.4637% S.D. = 0.511543 % R.S.D. = 0.509182
S_8 : 120 %	60	60.414	731214	100.69	
S_9 : 120 %	60	60.494	732165	100.823	

Table-8: Accuracy results of Tipiracil

Sample ID	Concentration (µg/ml)			% Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	32	32.195	505624	100.609	Mean= 100.7527% S.D. = 1.097575 % R.S.D.= 1.089375
S_2 : 80 %	32	31.915	501243	99.734	
S_3 : 80 %	32	32.613	512164	101.915	
S_4 : 100 %	40	40.668	638137	101.67	Mean= 100.5967% S.D. = 1.172714 % R.S.D.= 1.165758
S_5 : 100 %	40	39.738	623584	99.345	
S_6 : 100 %	40	40.310	632541	100.775	
S_7 : 120 %	48	48.181	755635	100.377	Mean= 100.0547%

S_8 : 120 %	48	48.085	754124	100.177	S.D. = 0.397865
S_9 : 120 %	48	47.813	749878	99.610	% R.S.D. = 0.397647

Observation: The mean recoveries were found to be 100.41, 100.66 and 100.963% for Trifluridine and 100.75, 100.59 and 100.05% for Tipiracil. The limit for mean % recovery[19] is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision[20] of each method was ascertained separately from the peak areas obtained by actual determination of five replicates of a fixed amount of drug. Trifluridine & Tipiracil. The percent relative standard deviations[21] were calculated for Trifluridine & Tipiracil are presented in the Table-9.

i) Repeatability

Table-9: Repeatability Results of Trifluridine and Tipiracil

HPLC Injection Replicates	AUC for Trifluridine	AUC for Tipiracil
Replicate – 1	613568	645214
Replicate – 2	613241	635241
Replicate – 3	625408	635424
Replicate – 4	617412	635987
Replicate – 5	612541	635216
Average	**616434**	**637416.4**
Standard Deviation	**5363.157**	**4370.055**
% RSD	**0.870029**	**0.685589**

Observation: The repeatability[22] study which was conducted on the solution having the concentration of about 50 µg/ml for Trifluridine and 40 µg/ml for Tipiracil (n =5) showed a %RSD of 0.870029% for Trifluridine and 0.685589% for Tipiracil. It was concluded that the analytical technique showed good repeatability.

ii) Intermediate precision / Ruggedness

Table-10: Ruggedness Results for Trifluridine

Conc. of Trifluridine (API) (µg/ml)	Observed Conc. of Trifluridine (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
40	40.02	0.36	40.06	0.86
50	49.87	0.45	50.26	0.37
60	59.13	0.65	59.62	0.76

Table-11: Ruggedness Results for Tipiracil

Conc. Of Tipiracil (API) (µg/ml)	Observed Conc. of Tipiracil (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
32	31.44	1.08	32.01	0.29
40	40.07	0.35	40.052	0.45
48	48.89	0.75	47.97	0.18

Observation: Intraday and interday studies[23-24] show that the mean RSD (%) was found to be within acceptance limit (≤2%), so it was concluded that there was no significant difference for the assay, which was tested within day and between days. Hence, method at selected wavelength was found to be precise.

Robustness: Robustness[25] is defined as the capacity of that method to be unaffected by even small deliberate changes that occur in the method parameters. The evaluation of robustness of a method is done by varying the chromatographic parameters such as pH, flow rate, organic phase proportions change, ionic strength etc., and determining any possible effect on the results obtained by that method.

Table-12: Result of Method Robustness Test for Trifluridine

Change in parameter	% RSD
Flow (0.8 ml/min)	0.78

Flow (1.2 ml/min)	0.62
More Organic	0.76
Less Organic	0.52
Wavelength of Detection (261 nm)	0.86
Wavelength of detection (257 nm)	0.54

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase[26] (±5%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Trifluridine (API).

Table-13: Result of Method Robustness Test for Tipiracil

Change in parameter	% RSD
Flow (0.8 ml/min)	1.03
Flow (1.2 ml/min)	0.28
More Organic	0.71
Less Organic	0.65
Wavelength of Detection (233 nm)	1.04
Wavelength of detection (229 nm)	0.96

Observation: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & organic phase (±5%) studied to determine the robustness of the method are also in favour of (Table-13, % RSD < 2%) the developed RP-HPLC method for the analysis of Tipiracil (API).

LOD: The limit of detection[27] (LOD) is the lowest concentration of analyte in a sample which can be detected, but not quantitated. LOD is a limit test that specifies whether an analyte is above or below a certain value. Signal-to-noise ratio of three-to-one is used to determine LOD.

$$L.O.D. = 3.3\ (SD/S).$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOD was found to be 0.06 µg/ml and 0.08 µg/ml for Trifluridine and Tipiracil respectively.

LOQ: The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Signal-to-noise ratio of ten-to-one is used to determine LOQ[28].

$$L.O.Q. = 10\ (SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Observation: The LOQ was found to be 0.18 µg/ml and 0.24 µg/ml for Trifluridine and Tipiracil respectively.

Assay: – Assay[29] refers to chromatography based purity assay where a compound of unknown activity or purity is compared to a reference standard with precisely determined bioactivity or purity.

$$\text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution

DT = Sample solution dilution

P = Working standard percentage purity

The assay was performed as explained in the previous chapter. The results which are obtained are following:

Table-14: Assay of TRIFLURIDINE & TIPIRACIL Tablets

Brand name of Tablets	Labelled amount of Drug (mg) Trifluridine / Tipiracil	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Lonsurf Tablet (Taiho Pharmaceutical Co., Ltd.)	20mg/8.19mg	19.896 (±0.28)/7.956 (±0.11)	99.5(±0.34) / 99.15(±0.12)

Observation: The assay of Lonsurf Tablet containing Trifluridine and Tipiracil was found to be 99.5% and Tipiracil was found to be 99.15%.

Forced Degradation Studies

The results of the forced degradation studies[30-31] indicated the specificity of the developed method that has been developed. Trifluridine and Tipiracil were stable only in acidic, basic and thermal stress conditions and photolytic stress conditions. The results of stability studies are given in the following Table-15.

Table-15: Results of Force Degradation Studies of Trifluridine and Tipiracil API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	67.43	32.57	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	80.63	19.37	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	99.07	0.93	100.00
UV (254nm)	24Hrs.	99.21	0.79	100.00

3% 24Hrs.69.6430.36

Hydrogen peroxide

100.00

IV. SUMMARY AND DISCUSSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Trifluridine and Tipiracil in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Trifluridine and Tipiracil indicated that the developed method is specific for the simultaneous estimation of Trifluridine and Tipiracil in the bulk and pharmaceutical dosage forms. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The specific Retention times for Trifluridine and Tipiracil are found to be 2.132 and 3.624. The tailing factors were found to be 1.1 and 1.2 with theoretical plates 2966 and 3076 for Trifluridine and Tipiracil. The %Recoveries was determined as 100.51% and 100.46% for Trifluridine and Tipiracil in Accuracy. The %RSD in Repeatability is 0.87 and 0.68 with Intermediate Precision are 0.48 and 0.72 for Trifluridine and Tipiracil in Precision. In Linearity, the correlation coefficient was found to be 0.999 and 0.999 for Trifluridine and Tipiracil. The LOD for Trifluridine and Tipiracil was 0.06 and

0.08 and LOQ for Trifluridine and Tipiracil are 0.18 and 0.24. The conclusion for forced degradation studies indicated that Trifluridine and Tipiracil were stable only in acidic, basic and thermal stress conditions and photolytic stress conditions.

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