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Validated reverse phase-HPLC method for simultaneous estimation of anti-neoplastic agents trifluridine and tipiracil in pure form and marketed pharmaceutical tablet dosage form

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Abstract
Analytical Method Development and Validation for Trifluridine and Tipiracil in bulk and Combine Dosage Form by RP-HPLC, New method was established for simultaneous estimation of Trifluridine and Tipiracil by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Trifluridine and Tipiracil by using Symmetry C18 5µm (4.6 x 150mm), flow rate was 1.0 ml/min, mobile phase ratio was Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v), detection wavelength was 260nm. The retention times of Trifluridine and Tipiracil were found to be 2.324mins and 4.314mins respectively. The % purity of Trifluridine and Tipiracil was found to be 99.865% and 99.658% respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study n Trifluridine and Tipiracil was found in concentration range of 0µg-36µg and 0µg-39µg and correlation coefficient (r2) was found to be 0.9995 and 0.9998, % recovery was found to be 100.280, %RSD for repeatability was 0.174 and 0.709, % RSD for intermediate precision was 0.093 and 0.937 respectively. The precision study was precise, robust, and repeatable. LOD value was 1.377 and 1.079, and LOQ value was 4.174 and 3.272 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Trifluridine and Tipiracil in API and Pharmaceutical dosage form.

Keywords: Trifluridine and Tipiracil, Method Development, Validation, Accuracy

Introduction
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 [2] 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. Trifluridine/Tipiracil is the combination of an antineoplastic pyrimidine analogue (Trifluridine[3]) 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. The IUPAC Name of Trifluridine is 1-[(2R, 4S, 5R)-4-hydroxy-5-(hydroxy methyl) oxolan-2-yl]-5-(trifluoro methyl) pyrimidine-2, 4-dione. The Chemical Structure of Trifluridine is follows.

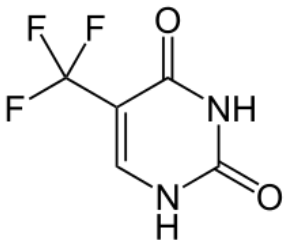

Fig 1: Chemical Structure of Trifluridine

Tipiracil 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 [4] (1+). 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 [5] 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. Tipiracil [6] is a Thymidine Phosphorylase Inhibitor. The mechanism of action of Tipiracil is as a Thymidine Phosphorylase Inhibitor. The IUPAC Name of Tipiracil is 5-chloro-6-[(2-imino pyrrolidin-1-yl) methyl]-1H-pyrimidine-2, 4-Dione. The Chemical Structure of Tipiracil is as following.

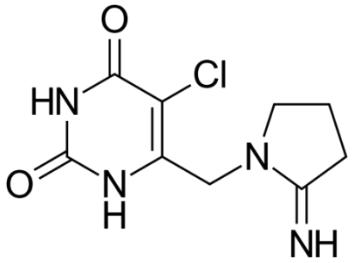

Fig 2: Chemical Structure of Tipiracil

The literature survey [36-40] revealed that Trifluridine and Tipiracil were estimated in bulk form and Marketed pharmaceutical dosage form by RP-HPLC. Moreover the Trifluridine and Tipiracil drugs were estimated by several methods. But the drugs were not estimated by any of the techniques, UV spectroscopy and RP-HPLC. Hence the present work was aimed to develop and validate an RP-HPLC method.

Experimental

Table 1: List of Equipments

S. No.	Instrument	Model No.	Software	Manufacturer's Name
1	HPLC Alliance	Waters 2695	Empower	Waters
2	UV Double Beam Spectrophotometer	UV 3000	UV Win 5	Lab India
3	Digital Weighing Balance	BSA224SCW	-	Sartorius
4	pH meter	AD102U	-	Lab India
5	Ultra Sonicator	SE60US	-	-
6	Suction Pump	VE115N	-	-

Table 2: List of Materials and Chemicals

S.No.	Chemical	Manufacturer	Grade
1	Water	Merck	HPLC Grade
2	Methanol	Merck	HPLC Grade
3	Acetonitrile	Merck	HPLC Grade
4	Potassium dihydrogen orthophosphate	Merck	A.R

Selection of Wavelength: The detection wavelength [7] 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.

Method Development

Preparations and Procedures

Preparation of Phosphate buffer: (pH: 3.8): Weighed 0.136086 grams of KH_2PO_4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 3.8 with ortho phosphoric acid.

Preparation of Mobile Phase: A mixture of pH 3.8 Phosphate buffer 600 mL (60%), 200 mL of MEOH (20%) and 200 mL of Acetonitrile are taken and degassed in ultrasonic water bath for 15 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration [8].

Diluent Preparation: Mobile phase is used as Diluent.

Preparation of the individual Trifluridine standard preparation: 10mg of Trifluridine working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent [9] is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of the individual Tipiracil standard preparation: 10mg of Tipiracil working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent. (Stock solution). Further 10.0ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of Sample Solution: (Tablet) Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Trifluridine and Tipiracil (marketed formulation) sample into a 10mL clean dry

volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted up to the mark with diluent.

Procedure: 20µL of the standard, sample are injected into the chromatographic system and the areas for Trifluridine and Tipiracil peaks are measured and the %Assay [10] is calculated by using the formulae.

Results and Discussion

Method Development

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. The overlay spectrum of Trifluridine and Tipiracil was obtained and the isobestic point of Trifluridine and Tipiracil showed absorbance's maxima [11] at 260 nm.

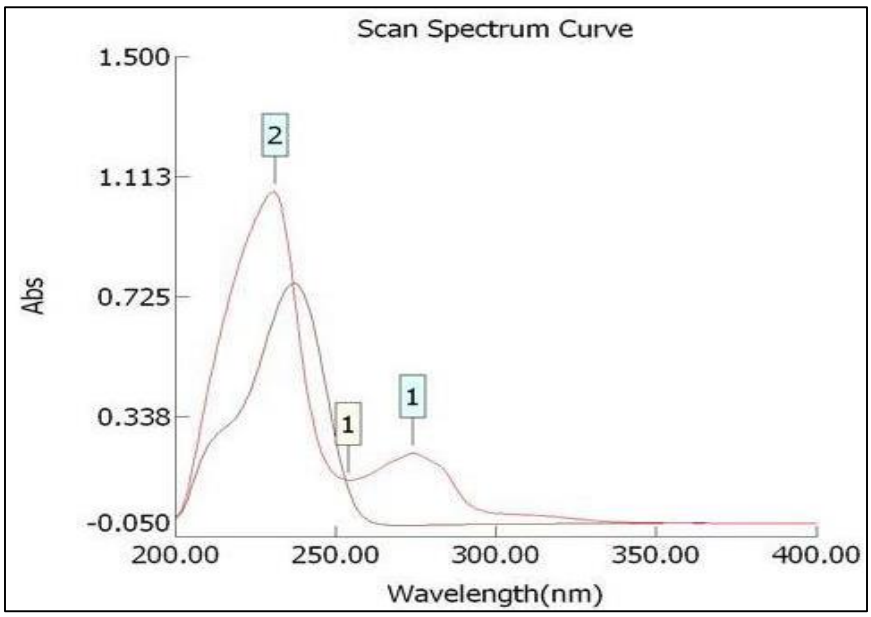

Fig 3: Overlay Spectrum for Trifluridine and Tipiracil

Optimization of Method

Optimized Chromatographic Conditions

Column : Symmetry C18 5µm (4.6 x 150mm)
Mobile phase ratio : Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v)
Detection wavelength : 260nm
Flow rate : 1.0ml/min
Injection volume : 20µl
Column temperature : Ambient
Auto sampler temperature: Ambient

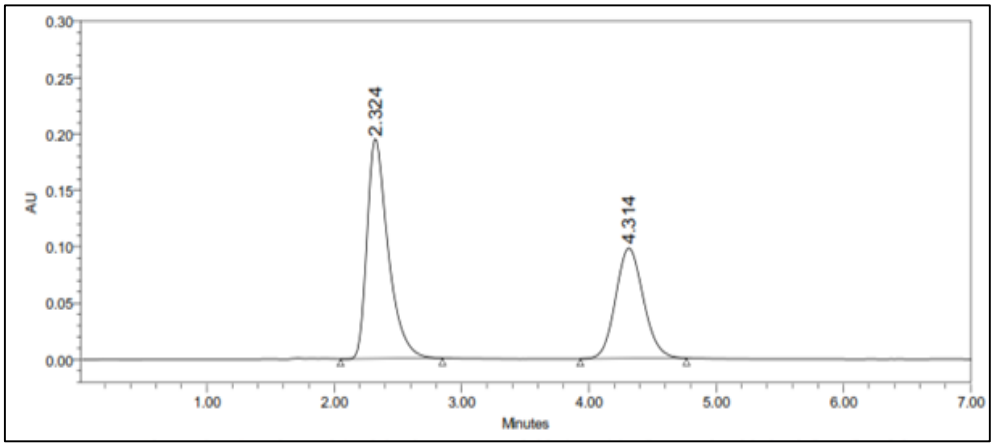

Fig 4: Chromatogram of Optimized Method

Method Validation

The method of analysis [12] was validated as per the recommendations of ICH4 and USP5 for the parameters like accuracy, linearity, precision, detection limit, quantitation and robustness [13].

System Suitability: System Suitability [14] was the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as tailing factor, resolution [15], plate count and reproducibility are determined and compared against the specification suitable for the method.

Table 3: Data of System Suitability Test for Trifluridine

S. No.	Injection No.	RT	Area	USP Plate Count	USP Tailing	Resolution
1	Injection 1	2.327	946257	5245	1.3	8.6
2	Injection 2	2.328	946325	5326	1.2	8.7
3	Injection 3	2.319	946859	5124	1.3	8.9
4	Injection 4	2.320	945875	5296	1.3	8.6
5	Injection 5	2.323	946396	5248	1.2	8.9
6	Injection 6	2.328	946548	5295	1.3	8.7
Mean			**946376.7**			
S.D			**325.8936**			
%RSD			**0.034436**			

Table 4: Data of System Suitability Test for Tipiracil

S. No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	4.331	112543	3854	1.4
2	Injection 2	4.341	111652	3965	1.5
3	Injection 3	4.299	112854	3874	1.3
4	Injection 4	4.313	111485	3698	1.5
5	Injection 5	4.325	113526	3785	1.4
6	Injection 6	4.341	112985	3965	1.6
Mean			**112507.5**		
S.D			**795.4945**		
%RSD			**0.707059**		

Linearity: Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity [16] is generally reported as the variance of the slope of the regression line [17].

Table 5: Chromatographic data for linearity study of Trifluridine

Concentration µg/ml	Average Peak Area
0	0
12	523864
18	764875
24	999874
30	1235658
36	1488542

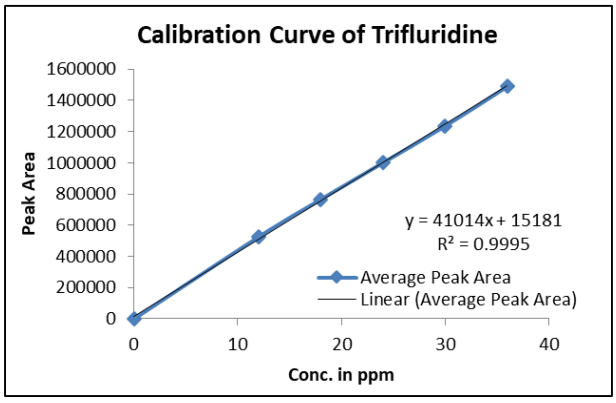

Fig 5: Calibration Curve of Trifluridine

Linearity Plot
The plot of Concentration (x) versus the Average Peak Area (y) data of Trifluridine is a straight line.

Y = mx + c
Slope (m) = 41014
Intercept (c) = 15181
Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient [18] is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 15181. These values meet the validation criteria.

Table 6: Chromatographic Data for Linearity Study of Tipiracil

Concentration µg/ml	Average Peak Area
0	0
13	65698
19.5	98254
26	128587
32.5	160648
39	191874

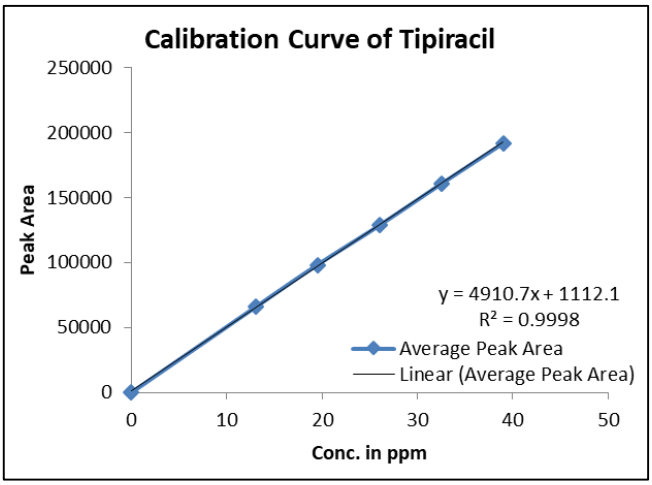

Fig 6: Calibration Curve of Tipiracil

Linearity Plot
The plot of Concentration (x) versus the Average Peak Area (y) data of Tipiracil is a straight line.

Y = mx + c
Slope (m) = 4910.7
Intercept (c) = 1112.1
Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 1112.1. These values meet the validation criteria.

Accuracy

Accuracy was confirmed by recovery studies by adding known amount of pure drug to the previously analyzed formulation by a proposed method. The % recovery [19] of Trifluridine and Tipiracil present in formulation was found between 98-102%. The% RSD values were found to be within the limits, it was extremely low when compared to the normal value. The high % recovery studies specify that there is no interference by the excipients present in the formulation, hence the described method was found to be accurate [20]. The values were given in table.no.7.

Table 7: Accuracy results of Trifluridine

%Concentration (at specification Level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean Recovery
50%	508367	12	12.024	100.200%	100.150%
100%	999100.3	24	23.989	99.954%	
150%	1496200.3	36	36.110	100.305%	

Table 8: Accuracy results of Tipiracil

%Concentration (at specification Level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	65093.67	13	13.029	100.223%	100.280%
100%	129339.3	26	26.111	100.426%	
150%	178242.7	39	36.070	100.194%	

Precision

The precision [21] of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.
Repeatability: Obtained five (5) replicates of 100% accuracy [22] solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 9: Results of repeatability for Trifluridine

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Trifluridine	2.321	946253	155465	5326	1.36	8.25
2	Trifluridine	2.317	947845	154578	5246	1.37	8.26
3	Trifluridine	2.323	945867	155845	5478	1.35	8.34
4	Trifluridine	2.322	948572	155698	5425	1.38	8.37
5	Trifluridine	2.324	949857	154857	5326	1.36	8.39
Mean			**947678.8**				
Std. Dev			**1649.66**				
%RSD			**0.174074**				

Table 10: Results of Repeatability for Tipiracil

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Tipiracil	4.304	111563	13254	3869	1.42
2	Tipiracil	4.300	111254	13425	3852	1.43
3	Tipiracil	4.308	111672	13254	3896	1.45
4	Tipiracil	4.310	112654	13265	3962	1.42
5	Tipiracil	4.314	113123	13154	3874	1.48
Mean			**112053.2**			
Std. Dev			**795.2614**			
%RSD			**0.709718**			

Intermediate Precision/Ruggedness

Table 11: Results of Intermediate precision for Trifluridine

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate Count	USPTailing	Resolution
1	Trifluridine	2.328	956325	156325	5246	1.35	8.24
2	Trifluridine	2.326	958741	157854	5367	1.38	8.26
3	Trifluridine	2.327	957542	156986	5265	1.34	8.47
4	Trifluridine	2.326	956895	158547	5384	1.39	8.29
5	Trifluridine	2.331	957486	156985	5297	1.35	8.34
Mean			**957397.8**				
Std. Dev.			**899.5091**				
% RSD			**0.093954**				

Table 12: Results of Intermediate precision for Tipiracil

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USPPlate count	USPTailing
1	Tipiracil	4.335	121231	13458	3896	1.52
2	Tipiracil	4.336	121457	13674	3785	1.54
3	Tipiracil	4.334	123142	13485	3969	1.58
4	Tipiracil	4.337	121325	13958	3859	1.57
5	Tipiracil	4.340	123654	13875	3789	1.59
Mean			**122161.8**			
Std. Dev.			**1145.733**			
% RSD			**0.937882**			

Method Robustness: Influence of small changes [23] in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase [24] (±2%) studied to determine the robustness of the method are also in favour of (Table-13, % RSD < 2%) the developed RP-HPLC method[25] for the analysis of Trifluridine (API).

Table 13: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.96
Flow (0.9 ml/min)	0.84
Temperature (27^{0}C)	0.81
Temperature (23^{0}C)	0.94
Wavelength of Detection (265 nm)	0.56
Wavelength of detection (255 nm)	0.17

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-14, % RSD < 2%) the developed RP-HPLC method for the analysis of Tipiracil (API).

Table 14: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.58
Flow (0.9 ml/min)	0.64
Temperature (27^{0}C)	0.72
Temperature (23^{0}C)	0.91
Wavelength of Detection (265 nm)	0.86
Wavelength of detection (255 nm)	0.78

Limit of detection (LOD) & Limit of quantification (LOQ)

The detection limit [26] (LOD) and quantitation limit (LOQ) may be expressed as:

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

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

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & Discussion: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 1.377µg/ml & 4.174µg/ml respectively for Trifluridine.

The LOD was found to be 1.079µg/ml and LOQ [27] was found to be 3.272µg/ml for Tipiracil which represents that sensitivity of the method is high.

Estimation of Trifluridine & Tipiracil in TABLET Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated [28] well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection (Blank Solution) of the standard solution [29] also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table 15.

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 [30] was performed as explained in the previous chapter (Above). The results which are obtained are following:

Table 15: 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	0.387 (±0.09) /0.485 (±0.08)	99.865 (±0.245) /99.658 (±0.354)

Result and Discussion: The assay of Lonsurf Tablets containing Trifluridine and Tipiracil was found to be 99.865% and Tipiracil was found to be 99.658%.

Forced Degradation Studies

Results of Degradation Studies: The results of the forced degradation studies [31-35] indicated the specificity of the developed method that has been developed. Trifluridine and Tipiracil were stable only in acidic and thermal stress conditions. The results of stability studies are given in the following Tables-16 & 17.

Table 16: Results of Force Degradation Studies of Trifluridine API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.316	12.684	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	78.155	21.845	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	86.215	13.785	100.00
UV (254nm)	24Hrs.	76.346	23.654	100.00
3% Hydrogen Peroxide	24Hrs.	75.104	24.896	100.00

Table 17: Results of Force Degradation Studies of Tipiracil API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	85.155	14.845	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	77.514	22.486	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	84.522	15.478	100.00
UV (254nm)	24Hrs.	74.251	25.749	100.00
3% Hydrogen Peroxide	24Hrs.	73.015	26.985	100.00

Summary and Conclusion

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for simultaneous analysis of Trifluridine and Tipiracil different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry C18 5µm (4.6 x 150mm) column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Trifluridine and Tipiracil it is evident that most of the HPLC work can be accomplished in the wavelength range of 240-300 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay & stability which can help in the simultaneous analysis of Trifluridine and Tipiracil in different formulations.

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