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RP HPLC method development and validation for simultaneous determination of trifluridine and tipiracil in pure and tablet dosage form

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ABSTRACT

In this research, Lonsurf Tablets (strength:Trifluridine 20mg and Tipiracil 8.19mg) was investigated for trifluridine and tipiracil content by isocratic RP-HPLC Procedure. Potent separation with reproducible resolution of trifluridine and tipiracil was brought using acetonitrile (35%) and 0.1M dipotassium hydrogen phosphate (65%) at 1.0ml/min rate of flow. 10 µl and 265 nm are the sample injection volume and wavelength set for detection and analysis , respectively. The procedure was validated and favourable linearity of Trifluridine (10 to 30 µg/ml) and Tipiracil (4.095 to 12.285 µg/ml) was achieved with regression coefficient of 0.9999 and 0.9998, respectively. The proposed procedure of RP-HPLC proved to be suitable, rapid, accurate, precise, selective and robust for partition and assessment of Trifluridine and Tipiracil in tablets in quality control labs.

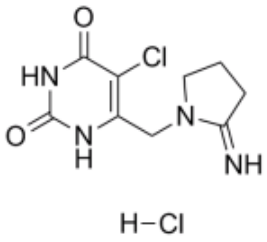
Keywords: Trifluridine, Tipiracil, RP-HPLC, Validation.

INTRODUCTION

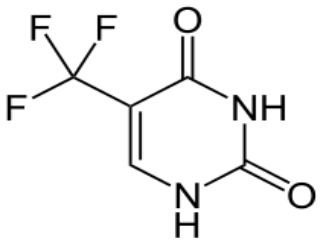
Trifluridine, is a fluorinated pyrimidine nucleoside that is structurally related to idoxuridine. It is an active antiviral agent in ophthalmic solutions used mainly in the treatment of primary keratoconjunctivitis abd recurrent epithelial keratitis due to herpes simplex virus.

Tipiracil, is a member of the class of pyrimidones that is uracil substituted by chloro and methyl groups. It has a role of anti neoplastic agent and an thymidine phosphorylase inhibitor. Tipiracil in combination with Trifluridine is used as the treatment for metastatic colorectal cancer. The mechanism of action of Trifluridine and Tipiracil hydrochloride (Lonsurf) is where the Trifluridne is phosphorylated and binds to thymidylate synthase enzyme active site. This enzyme was inhibited and reduces the nucleotide levels necessary for DNA replication. Also Tipiracil hydrochloride causes fragmentation of DNA through incorporation into DNA strands which results in tumour growth inhibition.

Chemical structure of Tipiracil Hydrochloride



Chemical structure of Trifluridine



MATERIALS AND METHODS

Chromatographic conditions

A prominent isocratic HPLC system –Model 2795 water alliance chromatography system with photodiode array detector. Column used for the development and validation of the method – develosil C18 (250x4.6mm),of particle size 5µm.HPLC Grade water, acetonitrile and dipotassium hydrogen phosphate. Isocratic mobile phase system with pH 3.8 comprising of acetonitrile (35%) and 0.1M dipotassium hydrogen phosphate (65%) at 1.0ml/min rate of flow. 10 µl is the sample injection volume. Temperature in the column was set to 25°*c*.The wavelength was set to 265nm for detection and analysis.

Preparation of mobile phase

Prepared by combining acetonitrile and 0.1M dipotassium hydrogen phosphate in the ratio 65:35(v/v) were mixed and degassed In a digital ultrasonicator for 10minutes.The same is employed for preparing standard solutions.

Standard stock solution of Trifluridine and Tipiracil

Standard stock solution of Trifluridine and Tipiracil of concentration of 200µg/ml and 81.9µg/ml,respectively was made by dissolving 20mg of trifluridine and 8.19mg of tipiracil in 100ml of mobile phase.

Standard working solution of Trifluridine and Tipiracil

Stock solution of tipiracil and trifluridine of concentration of 81.9 µg/ml and 200 µg/ml, respectively was made by dissolving 8.19 mg (tipiracil) and 20 mg (trifluridine) in 100 ml of mobile phase. Calibration solutions (CS) were made as below:

- CS 1: 0.5 ml stock solution is made up to 10 ml with mobile phase. Concentration: tipiracil – 4.095 µg/ml and trifluridine – 10 µg/ml
- CS 2:0.75 ml stock solution is made up to 10 ml with mobile phase. Concentration: tipiracil – 6.1425 µg/ml and trifluridine – 15 µg/ml
- CS 3: 1.0 ml of stock made up to 10 ml with mobile phase. Concentration: tipiracil – 8.19µg/ml and trifluridine – 20 µg/ml
- CS 4:1.25 ml stock solution is made up to 10 ml with mobile phase. Concentration: tipiracil – 10.2375 µg/ml and trifluridine – 25 µg/ml
- CS 5: 1.5 ml stock solution is made up to 10 ml with mobile phase. Concentration: tipiracil – 12.285 µg/ml and trifluridine – 30 µg/ml

Validation solution was made by diluting 1.0 ml of stock to 10 ml with mobile phase.

Calibration Curves of Trifluridine and Tipiracil

Calibration solutions of 1 to 5 were introduced into the system in uphill order of Trifluridine and Tipiracil Concentrations. Optimised conditions of

HPLC were employed. The areas of Trifluridine aand tipiracil peaks in calibration solutions 1 to 5 are determined. Calibration curves are produced using the areas of Trifluridine and Tipiracil peaks to that of concentrations of Trifluridine and Tipiracil. Regression equation for Trifluridine and Tipiracil was calculated.

Assay of Trifluridine and Tipiracil in Tablets

Precisely weigh and transfer powdered Lonsurf tablets equal to Trifluridine 20mg and Tipiracil 8.19mg to a standard flask of 100ml capacity. The contents are macerated in 20ml mobile phase (sonicated 20min using ultra sonicator to confirm complete extraction).The filtered mobile phase extract was diluted to mark with mobile phase. The sample solution was filtered 0.45µm membrane filter. The test solution was made by diluting 1.0ml of above prepared solution to 10ml with mobile phase(concentration:Tipiracil-8.19µg/ml and Trifluridine 20µg/ml) and analysed by HPLC procedure.

Method development

For developing a method for estimation of Trifluridine and Tipiracil in pure tablet dosage form and bulk drug, different parameters were tried to elute both drugs with good resolution, less tailing factor and greater plate count. Satisfactory results obtained from given chromatographic conditions for Trifluridine and Tipiracil in the given table no.1

Table 1: Method Validation parameters

Parameters	Chromatographic Conditions
Mobile phase	K2HPO4(0.M):Methanol(65:35)
Column	Develosil,c18,250x4.6mm,5µm
Flow Rate	1ml/min
Temperature	25°c
Volume Injected	10µl
Time of Run	10min
Detection	265nm

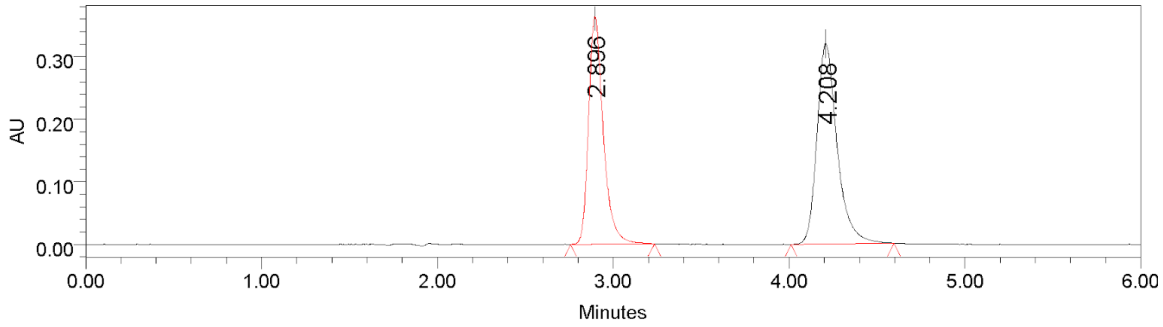


Fig 1: Chromatogram of Trifluridine and Tipiracil

Method validation

The HPLC method suggested was validated for system suitability, selectivity, linearity, sensitivity, precision, robustness and accuracy according to rules of ICH.

Linearity

Peak areas of Tipiracil and Trifluridine calibration solutions 1 to 5 were determined. The method proved linearity from 4.095 to 12.285µg/ml for Tipiracil and 10 to 30µg/ml for Trifluridine. Linear regression data for Tipiracil and Trifluridine is a indicative of good linearity.

Table 2: Linear response data of trifluridine and tipiracil

Trifluridine concentration in µg/ml units	Trifluridine peak area in mAU units	Tipiracil concentration in µg/ml units	Tipiracil peak area in mAU units
10	1040321	4.095	1255711
15	1564510	6.14	1882716
20	2088850	8.19	2515326
25	2604442	10.2375	3145761
30	3124462	12.29	3772196

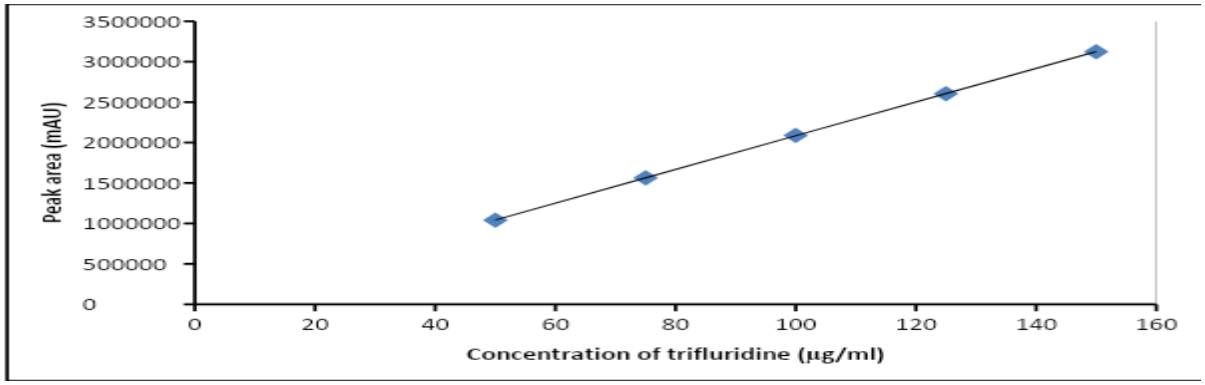


Fig 2: Linearity response curve of Trifluridine

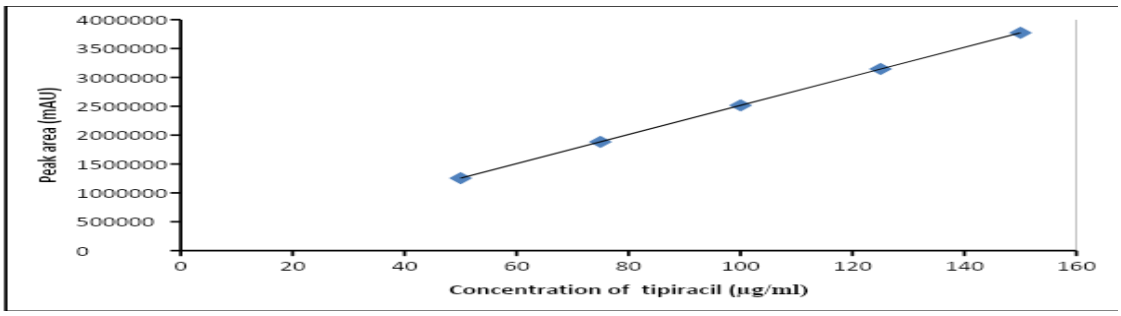


Fig 3: Linearity response curve of Tipiracil

Limit of detection & limit of quantification

Signal-to-noise scale of 3 was determined for LOD (tipiracil – 0.032 µg/ml and trifluridine – 0.070 µg/ml) and Signal-to-noise scale of 10 was determined for LOQ (tipiracil – 0.175 µg/ml and trifluridine – 0.233 µg/ml).

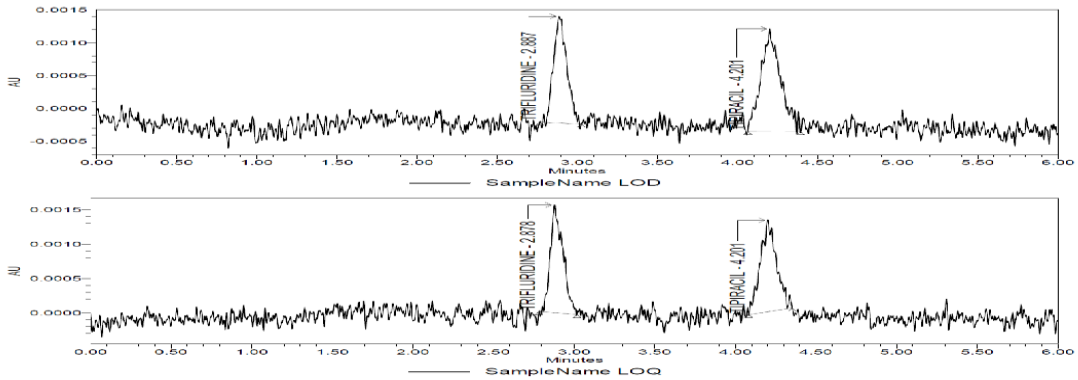


Fig4: LOD and LOQ level chromatograms

Sample Name	Peak Name	RT	AREA	S/N
LOD	**TRIFLURIDINE**	2.887	9513	3.71
LOQ		2.878	8458	10.54
LOD	**TIPIRACIL**	4.201	13314	3.60
LOQ		4.201	9398	10.02

Precision

Precision for tipiracil and trifluridine estimation was done at concentration: tipiracil – 8.19 µg/ml and trifluridine – 20 µg/ml. The precision was expressed as standard deviation and relative standard deviation of six tipiracil and trifluridine peak areas. The small relative standard deviation showed preciseness of method.

Table 3: Precision of tipiracil and trifluridine

Serial no.	TRIFLURIDINE PEAK AREA	TIPIRACIL PEAK AREA
1	2088842	2517816
2	2087186	2517678
3	2083027	2518543
4	2088739	2513376
5	2085261	2517785
6	2087392	2519738
Average	2086741	2517489
Standard Deviation	2236.615	2159.442
Relative Standard Devaition	0.107	0.086

Accuracy

Method accuracy was determined by weighing recoveries of tipiracil and trifluridine using method of standard addition. Tipiracil and trifluridine known quantities were fortified to pre-determined sample tablet solution of tipiracil and trifluridine at levels 50%, 100% and 150%. The results demonstrated good accuracy with good recovery of tipiracil and trifluridine.

Table 4: Recovery of trifluridine

	Trifluridine fortified (µg/ml)	Trifluridine determined (µg/ml)	Trifluridine recovered (%)	Trifluridine average recovered (%)
	10	10.04	100.40	100.01
50%	10	9.99	99.90	
	10	9.97	99.73	
100%	20	19.98	99.89	99.75
	20	19.91	99.56	
	20	19.96	99.80	
150%	30	29.95	99.82	99.74
	30	29.94	99.81	
	30	29.87	99.58	

Table 5: Recovery of tipiracil

	Tipiracil fortified (µg/ml)	Tipiracil determined (µg/ml)	Tipiracil recovered (%)	Tipiracil average recovered (%)
50%	4.095	4.05	99.00	99.22
	4.095	4.08	99.69	
	4.095	4.05	98.96	
100%	8.190	8.14	99.40	99.40
	8.190	8.14	99.41	
	8.190	8.14	99.38	
150%	12.285	12.25	99.70	99.66
	12.285	12.25	99.70	
	12.285	12.23	99.57	

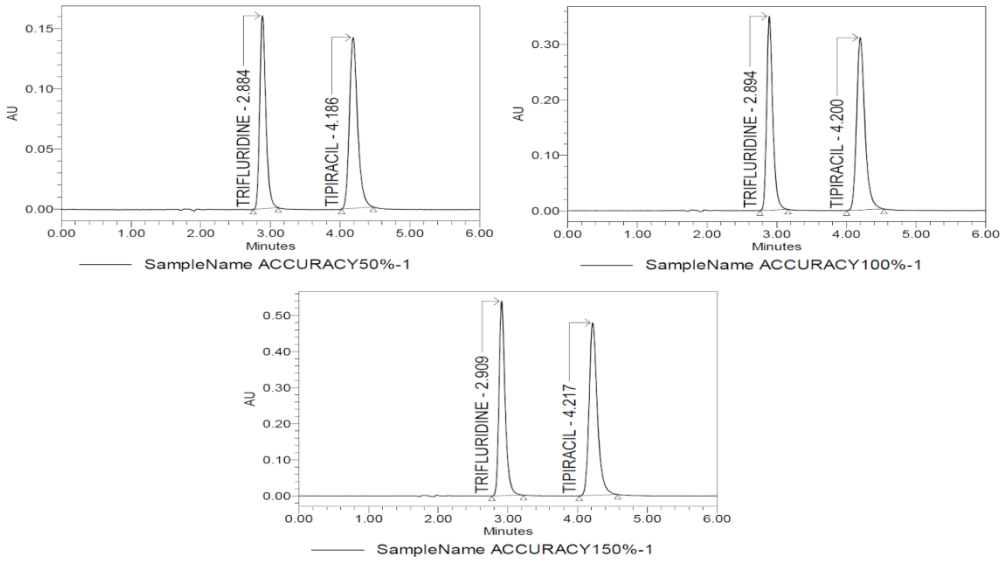


Fig 5: Accuracy chromatograms of trifluridine and tipiracil

Robustness

Method robustness was checked by performing the analysis using conditions throughout that mobile phase component (± 5% change in ratio of acetonitrile), flow rate (± 0.1 ml/min), temperature in column (± 2 ^{o}C) and pH in mobile phase (± 0.1 units) were modified and the result on the plate count, tailing factor and resolution were noted. The values are found in the limits of acceptance.

Table 6: Robustness of trifluridine and tipiracil

Condition	Trifluridine		Tipiracil		
	Peak tailing	Plate count	Peak tailing	Plate count	Resolution
Flow rate – 0.9 ml/min	1.26	4962	1.23	5777	6.64
Flow rate – 1.1 ml/min	1.25	5215	1.24	5921	6.73
Temperature - 23˚C	1.30	5844	1.26	6761	7.10
Temperature - 27˚C	1.27	6268	1.22	7058	7.26
pH – 3.7	1.32	5874	1.30	6691	7.14
pH – 3.8	1.32	5672	1.29	6555	7.00
Acetonitrile – 30%	1.26	4962	1.23	5777	6.64
Acetonitrile – 40%	1.30	5844	1.26	6761	7.10

Selectivity

Ten µl of standard solution (tipiracil – 8.19 µg/ml and trifluridine – 20 µg/ml), tablet sample solution (tipiracil – 8.19 µg/ml and trifluridine – 20 µg/ml) placebo without drugs and mobile phase without drugs were introduced in to the system. On comparing the respective chromatograms, no interfering peaks were seen at tipiracil and trifluridine peak retention times. So, proved method selectiveness for the assay of tipiracil and trifluridine.

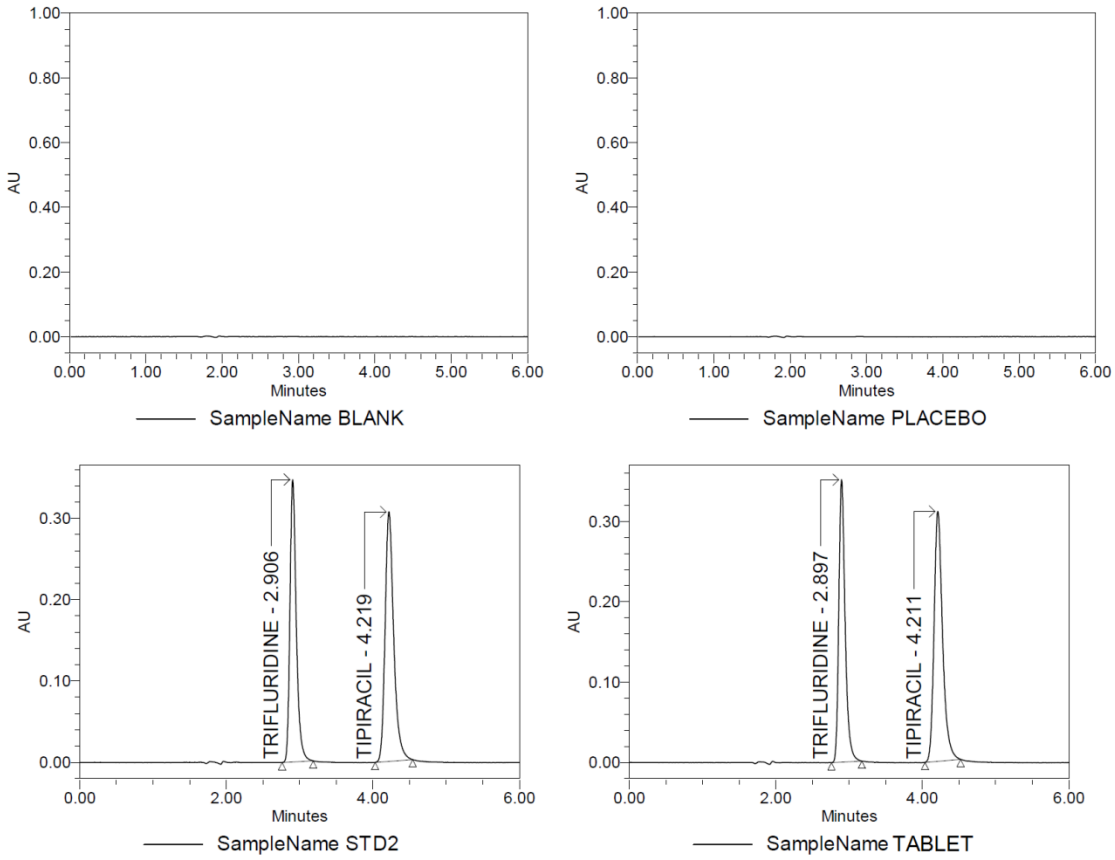


Fig 6: Selectivity chromatograms for trifluridine and tipiracil

System suitability

Tipiracil (8.19 µg/ml) and trifluridine (20 µg/ml) validation solution is introduced five times. Optimized chromatography conditions were used. Tailing in peak, plate count, resolution between tipiracil and trifluridine and relative standard deviation for peak areas tipiracil and trifluridine were established. Acceptance criteria for system suitability: Resolution - over 2.0; Plate count - over 2000; RSD for peak area – less than or upto 2.0; and Peak tailing - less than or upto 2.0.

Table 7: Suitability of system for trifluridine and tipiracil assay

Peak Name: TRIFLURIDINE

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	STD2	TRIFLURIDINE	2.906	2074485	5887	1.31
2	STD2	TRIFLURIDINE	2.907	2087160	5771	1.32
3	STD2	TRIFLURIDINE	2.906	2084206	5751	1.31
4	STD2	TRIFLURIDINE	2.897	2099185	5811	1.33
5	STD2	TRIFLURIDINE	2.896	2085441	6251	1.33
Mean				2086095.4		
% RSD				0.4		

Peak Name: TIPIRACIL

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing
1	STD2	TIPIRACIL	4.221	2527112	6595	7.08	1.29
2	STD2	TIPIRACIL	4.220	2537481	6613	7.07	1.32
3	STD2	TIPIRACIL	4.219	2501243	6608	7.05	1.28
4	STD2	TIPIRACIL	4.211	2512326	6645	7.11	1.29
5	STD2	TIPIRACIL	4.208	2520597	7023	7.33	1.31
Mean				2519751.7			
% RSD				0.5			

Estimation of Trifluridine and Tipiracil in Tablets

10 µl of test sample (tipiracil – 8.19 µg/ml and trifluridine – 20 µg/ml) prepared was applied three times to HPLC. It was developed and detected using the procedure defined. The results of peak areas obtained corresponding to tipiracil and trifluridine were used for quantification in tablet samples by calibration curve or regression equation. The results of three analyses were expressed as mean amount of tipiracil and trifluridine in mg content per tablet. The recovery and less relative standard deviation values of tipiracil and trifluridine determined the fitness of the procedure for routine quality control studies of tipiracil and trifluridine.

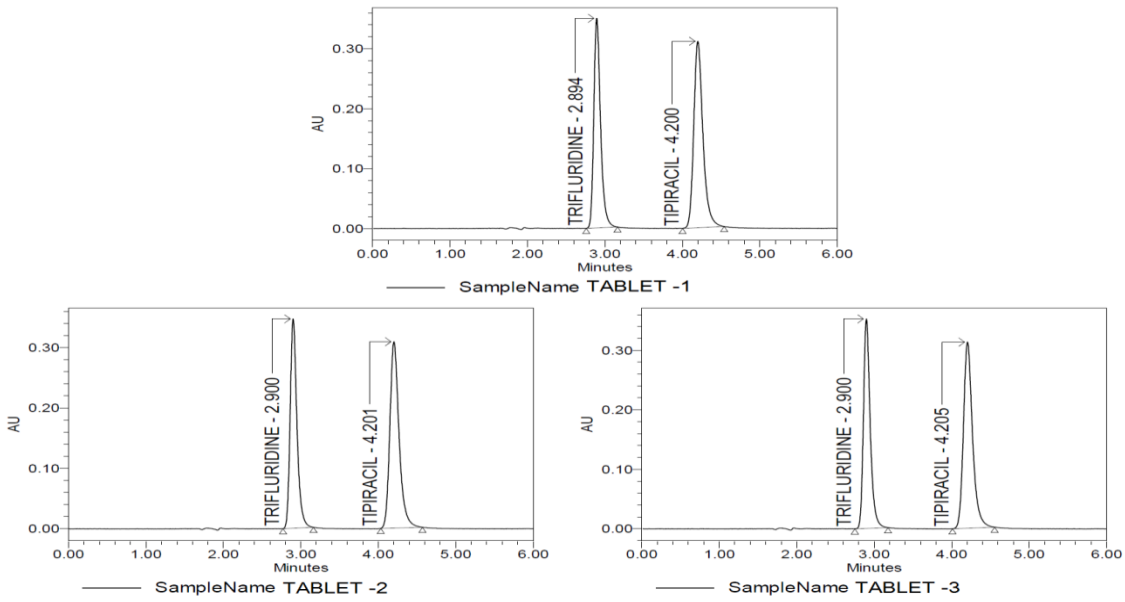


Fig 7: Trifluridine and Tipiracil assay in tablet chromatograms

Table 8: Trifluridine and Tipiracil assay in tablet

Labeled drug claim in tablet (mg)	Determined content of drug (mg)	Statistics
Trifluridine		
20	20.002	Average: 19.97 mg
20	19.95	SD: 0.031%
20	19.948	RSD: 0.153%
Tipiracil		
8.19	8.126	Average: 8.14 mg
8.19	8.141	SD: 0.018%
8.19	8.162	RSD: 0.222%

RESULTS AND DISCUSSION

The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Trifluridine and Tipiracil in bulk drug and pharmaceutical dosage form by using Develosil C18 column with photodiode array detection. The run time was set at 10minutes and the retention time for Trifluridine and Tipiracil was 2.896 & 4.208 respectively. The LOD & LOQ values were found to be 0.070 & 0.233 µg/ml for Trifluridine and 0.032 & 0.175 µg/ml for Tipiracil. Linearity of Trifluridine and Tipiracil was in range of concentration 10-30 µg/ml. For precision, %RSD were found to be 0.107% (Trifluridine) and 0.086% (Tipiracil). The percent recoveries were 99.74%-100.01% and 99.22%-99.66% for Trifluridine and Tipiracil respectively.

CONCLUSION

This investigation describes a rapid and robust HPLC procedure for determination of tipiracil and trifluridine at the same time in their tablet dosage forms. The proposed procedure have the ICH acceptance validation criteria related to system suitability, linearity, selectivity, robustness, sensitivity, accuracy and precision.

Acknowledgement

I take privilege to sincerely thank to my professor for her extreme support and guidance all throughout my work.

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