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Development and Validation of Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Trifluridine and Tipiracilin Bulk and their Combined Dosage form

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Abstract : The present work describes Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of Trifluridine and Tipiracilin bulk and their combined dosage form. The chromatographic separation was performed on Column :XterraC_{18} (150mm x 4.5mm x 5µ) using Trietylamine buffer: Acetonitrile (40:60) as mobile phase at a flow rate of 1 mL/min and column oven temperature of 30ºC. The detection was carried out using a Diode array detector at 272 nm. Total run time was 10 minutes within which main compounds and their degradation products were separated. The method was validated for accuracy, repeatability, reproducibility, robustness, linearity, limit of detection and quantification were established. The developed method was successfully applied to the simultaneous quantitative analysis of the title drugs in tablet dosage forms.

Keywords : Stability indicating assay, RP-HPLC, Trifluridine and Tipiracilin, Forced degradation studies.

Introduction

Trifluridine:

chemically 1-[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-5-(trifluoromethyl)-1,2,3,4-tetra hydropyrimidine-2,4-dione[1].Trifluridine is metabolized by the enzyme thymidine phosphorylase to 5-trifluoromethyl-2,4(1H,3H)-pyrimidinedione (FTY), and also by glucuronidationis. It has a molecular formula of $C_{10}H_{11}F_3N_2O_5$ and a molecular weight- 296.19 g/mol, Freely soluble in water, Freely soluble in methanol and in ethanol Slightly soluble in diethyl ether. It has the following structural formula as shown in fig.1a

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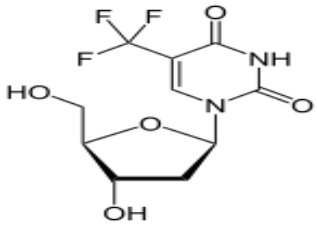


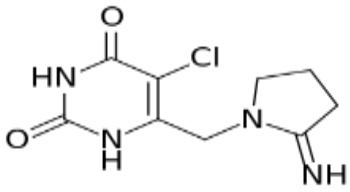


Fig.1a. Structure of Trifluridine **Fig. 1b. Structure of Tipiracil**

Tipiracil:

chemically 5-Chloro-6-[(2-imino-1-pyrrolidinyl)methyl]-2,4(1H,3H)-pyrimidinedione[2].Tipiracil is a thymidine phosphorylase (TPase) inhibitor and inhibits degradation of trifluridine by inhibiting TPase, thus increasing systemic exposure to trifluridine when tipiracil is given together with trifluridine.. It has a molecular formula of C9H11ClN4O2 and a molecular weight- 242.67g/mol, freely soluble in water, NaOH,HCL Slightly soluble in methanol and in ethanol practically insoluble in acetone. It has the following structural formula as shown in fig.1b.

The literature survey reveals that there are few HPLC and spectroscopic methods available for the determination individual Trifluridine and Tipiracil in bulk and dosage forms. Different acivity were developed by using crude extracts of plants[3-10]. There were no reported analytical methods for simultaneous estimation Trifluridine and Tipiracil in bulk and their combined dosage forms in presence of their degradation products. Hence an author made an attempt to develop stability indicating specific, sensitive, accurate and precise RP-HPLC method for simultaneous estimation of these drugs. The developed method was validated as per ICH Q2 guidelines.

Materials and Methods

Chemicals and reagents:

Working standards of Trifluridine and Tipiracil were obtained as gift samples from NATCO Pharma, Hyderabad, India. HPLC grade Acetonitrile was purchased from Merck (Mumbai, India), HPLC grade Water (Milli Q or equivalent) all chemicals (AR Grade) were used for entire study.

Instrumentation:

All HPLC experiments were carried out on a Waters Alliance 2690 separation module, with waters 2996 photodiode array detector using Auto sampler. Data collection and processing was done using Empower PDA 2 software. The analytical column used for the separation was XterraC_{18}, 150mm x 4.5mm, 5µm Column, Other equipments used were ultra-sonicator (Remi), Analytical balance (OHUAS) P^H meter (EU TECH).

Preparation of solutions:

Buffer: Mix 1ml Triethyl amine in 1litre water adjust pH-3 with OPA.
Mobile phase: Mix Acetonitirile and Buffer in the ratio of 60+40.Filter through 0.46lµ membrane filter paper.
Diluent: Mobile phase is used as diluents.

Preparation of the Trifluridine & Tipiracil Standard & Sample Solution:

Standard Solution Preparation:

Accurately weigh and transfer 20mg & 9mg of Trifluridine & Tipiracil working standard into a 10ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1.0 ml of Trifluridine & Tipiracil of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.Further pipette 1.5 ml of Trifluridine & Tipiracil of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Method Development:

The optimized chromatographic conditions (fig no-6.13).The best peak shape and maximum separation was achieved with mobile phase composition of Acetonitrile and Buffer using (60:40) ,peak symmetry and reproducibility were obtained on XterraC_{18},150mm x 4.6mm, 5µm Column. The optimum wavelength for detecting the analytes was found to be 272nm, a flow rate of 1ml/min yielded optimum separation and peak symmetry. The optimized chromatographic conditions were shown table 1 and fig.2.

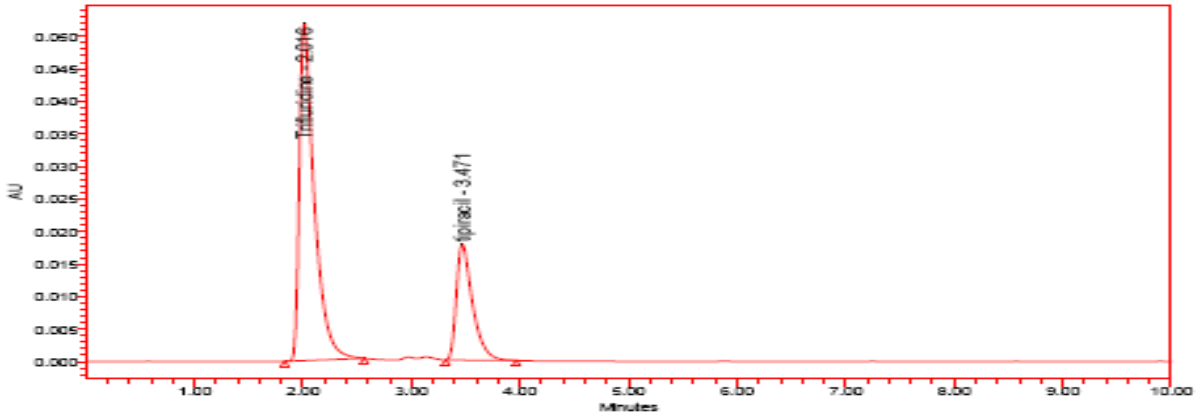


Fig.2: A Typical Chromatogram of TFD and TPR (optimized)

Table.1. Optimized Chromatographic Conditions:

Column	Xterra C18,4.5 × 150µm
Mobile phase	ACN+Buffer (TEA) (60+40)
Flow rate	1 mL /min
Column temperature	30°C
Injection volume	10 µL
Detection Wavelength	272 nm
Run time	10 minutes
Retention time	2.016(TFD),3.471(TPR)min

Table.2. Linearity Studies of Proposed Method:

Parameters	**Trifluridine**	**Tipiracil**
Linearity range (µg/ml)	10-50(µg/ml)	4.5-22.5(µg/ml)
Regresion equation	y = 34215x - 14106	y = 30967x – 9559.5
Slope	34215	30967
Intercept	14106	9559.5
Correlation coefficient (r)	0.999	0.999
LOD (µg/ml)	0.090	0.162
LOQ (µg/ml)	0.360	0.446

To saturate the column, the mobile phase was pumped for about 30 minutes thereby to get the base line corrected. The separate standard calibration lines were constructed for each drug. A series of aliquots were prepared from the above stock solutions using diluent to get the concentrations 4.5-22.5µg/ml for Tiiracil, 10-50µg/ml for Trifluridine. Each concentration 6 times was injected in to chromatographic system. Each time peak area and retention time were recorded separately for all the drugs. Calibration curves were constructed as by taking average peak area on Y-axis and concentration on X-axis separately for both drugs. From the calibration curves regression equations were calculated, these regression equations were used to calculate drug content in formulation. The obtained results were shown table. 2.

Estimation of Trifluridine and Tipiracil in tablet dosage forms:

Weigh 20 tablets and crush to powdered then take accurately weigh and transfer equivalent to 20mg & 9mg of Trifluridine &Tipiracil sample into a 10ml clean dry volumetric flask add about 7ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 1.0 ml of Trifluridine & Tipiracil of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.Further pipette 1.5 ml of Trifluridine & Tipiracilthe above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. This solution was estimated by above developed method. The assay procedure was repeated 6 times (n=6) the drug content was estimated using above calculated regression equation; the results of tablet dosage form are shown in the table 3.

Table.3. Results of Tablet dosage form:

Compound name	**Brand name**	**Label claim (mg)**	**Test concentration (µg/ml)**	**Mean amount estimated (µg/mL) (n=6)**	**%Assay**	**%RSD**
Trifluridine Tipiracil	Lansurf	20 8.9	30 13.5	30.105 13.61	100.35 100.68	0.362 0.095

Method Validation:

The analytical method was validated for various parameters as per ICH guidelines

Linearity:

The linearity of the method was determined in concentration range of 4.5-22.5 µg/ml for Tipiracil, 10-50 µg/ml for Trifluridine. Each solution was injected in triplicate. The average peak area versus concentration data of both drugs was treated by least squares linear regression analysis and the results obtained from as shown in tables 2.

Specificity and Selectivity:

Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by examining blank matrix samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other placebos. Two different samples were injected and studied with respective placebos. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and placebos) showed almost no interfering peaks with in retention time ranges. Fig.2shows the chromatograms for Trifluridine and Tipiracil with Blank and Placebo. The figures shows that the selected drugs were cleanly separated. Thus the HPLC method proposed in this study was selective.

Accuracy :

Accuracy is expressed as the closeness of the results from standard samples to that of the actual known amounts to determine the accuracy of the proposed method, recovery studies were carried out in different recovery levels (50%, 100% and 150%) by adding placebo to the pre-analyzed formulation .the solutions were suitably diluted in the range and then each of the dilution was observed 6 times. The% recoveries of the drugs were shown in the table 4.

Table.4. Recovery studies of Trifluridine and Tipiracil:

Drug	% Level of recovery	Pre analysed conc (ug/ml)	Amount found (ug/ml)	% Recovery	%RSD
Trifluridine	50% 100% 150%	10 20 30	10.06 20.02 30.25	100.61 100.10 100.83	0.4 0.2 0.5
Tipiracil	50% 100% 150%	1.28 2.56 4.14	1.29 2.57 4.14	100.8 100 100.7	0.6 0.4 0.3

Precision:

Precision is the degree of repeatability of an analytical method under normal operation conditions.

Precision is of 3 types

1. System precision
2. Method precision
3. Intermediate precision
   a. Intraday precision
   b. Inter day precision

Method precision was achieved by repeating the same procedure of preparation solution six times and injecting.System precision is checked by using standard chemical substance to ensure that the analytical system is working properly. In this peak area and % of drug of six determinations is measured and % RSD should be calculated.

In method precision, a homogenous sample of single batch should be analyzed 6times. This indicates whether a method is giving constant results for a single batch. In this analyze the sample six times and calculate the % RSD and the results are shown in the table 5.

Table. 5. Method Precision & System Precision studies of Trifluridine and Tipiracil

S.No.	Method Precision		System Precision	
	Trifluridine	**Tipiracil**	**Trifluridine**	**Tipiracil**
1	526748	198737	523413	193392
2	524262	198367	523308	194410
3	529843	197863	529589	196670
4	527634	198567	521637	199213
5	527859	196739	523346	195040
6	528674	195783	519999	193701
Mean	527503.3	197676	523548.7	195404.3
Standard deviation	1900.8	1174.2	3251.4	2199.1
%RSD	0.4	0.6	0.6	1.1

LOD and LOQ:

LOD:

It is lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated, experimental conclusions. The detection limit is usually expressed as the concentration of analyte. The standard deviation and response of the slope.

LOD = 3.3*signal /noise

LOQ:

The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The standard deviation and response of the slope and the results obtained.

LOQ= 10* signal /noise

Robustness:

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated. During robustness testing each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0 ± 0.1 ml/min), organic composition and wavelength (227±2) and the results obtained from as shown the table 6.

Table. 6. Robustness studies of Trifluridine and Tipiracil:

Method Parameters	Conditios	Retention Time(R_t)		Area		%Recovery	
		TFD	TPR	TFD	TPR	TFD	TPR
Flow +	+10 %	1.908	3.311	487789	180371	100.2	100.1
Flow -	-10 %	2.423	3.892	504492	183145	100.4	100.2
Organic phase+	+10%	1.912	3.298	487765	180654	100.1	100.3
Organic phase-	-10%	2.403	3.901	504492	183763	100.2	100.2
Wavelength +	+2 nm	4.905	3.519	2838384	824514	100.1	100.3
Wavelength -	-2 nm	4.905	3.519	2834246	827832	100.2	100.1

Table. 7. System Suitability Parameters:

Parameter	Trifluridine	Tipiracil	Acceptance criteria
Retention time	2.005	3.408	For information
Plate count	2117.66	2730.77	NLT 2000
Tailing	1.08	1.62	NMT 2
Resolution	0	5.74	NLT 1.5

System Suitability Parameters:

For assessing system suitability, six replicates of working standards samples of Trifluridine and Tipiracil.were injected and studied the parameters like plate number(N), tailing factor(K), resolution, relative retention time and peak asymmetry of samples. The results were tabulated in table 7.

Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitTPR stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Trifluridine and Tipiracil using the proposed method.

Preparation of stock:

Accurately weigh and transfer 20mg and 9mg of Trifluridine and Tipiracil working standard into a 10ml clean dry volumetric flask add diluent and sonicate to dissolve it completely and make volume up to the

mark with the same solvent (Stock solution)**.** Further pipette 1ml of Trifluridineand Tipiracil of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Hydrolytic degradation under acidic condition:

Pipette 1.5 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition: Pipette 1.5 ml of above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Thermal induced degradation:

Trifluridine and Tipiracilsample was taken in petridish and kept in Hot air oven at 110^0C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

Oxidative degradation:

Pipette 1.5ml above stock solution 2 into a 10ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials.

Solution stability:

The stock solution showed no significant change in analyte composition, retention time and peak areas of Trifluridine and Tipiracil1 weeks of storage at room temperature. This was sufficient for the whole analytical process.

Results and Discussion:

Optimized chromatographic conditions:

Most of all reported HPLC methods till date use C-8 or C-18 columns. Most of these use Complex mobile phase compositions. Hence, attempts were directed towards development of a Simple and better method on commonly used XterraC_{18} column with good resolution. Different logical Modifications were tried to get good separation among the drugs and the degraded products. These changes included change in mobile phase composition in isocratic elution as well as gradient modes on different HPLC columns.

Linearity, LOD and LOQ:

The calibration plot was linear over the concentration range investigated (4.5-22.5 µg/ml) for Tipiracil (10-50 µg/ml) for Trifluridine respectively (figure 6.14 &6.15). Average correlation coefficient R^2=0.999 for all the drugs with %RSD values ≤2.0 across the concentration ranges studied, was obtained from regression analysis. The LOD that produced the requisite precision and accuracy was found to be 0.25µg/ml Trifluridine and 0.0625 µg/ml Tipiracil drug. The resultant %RSD values were ≤1.00 %(table no.6.14).The LOQ for Trifluridine andTipiracil were found to be 0,505 µg/ml and 0.125 µg/ml respectively. The Regression results indicate that method was linear in the concentration range studied and can be used for detection and quantification of Trifluridine and Tipiracil in a very wide concentration range.

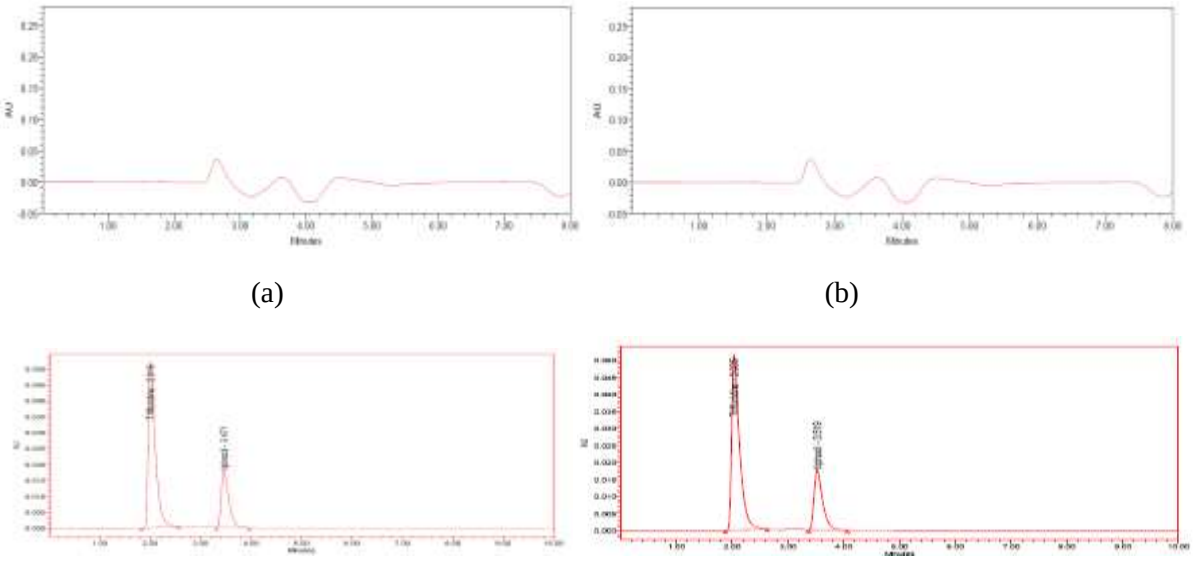
Accuracy and Precision:

Accuracy as recovery was evaluated by spiking previously analyzed test solution with additional Placebo at three different concentration levels (table-6.15). Recovery of previously analyzed test solution drug concentration added was found to be 100.51% for Trifluridine and 99.78% for Tipiracil with the value of RSD less than 1% indicating that the proposed method is accurate for the simultaneous estimation of all drugs from

their combination drug products in presence of their degradation products. The low RSD values indicate the repeatability and reproducibility of the Method (table-4 & 5).

Specificity and Selectivity:

Specificity is checked in each analysis by examining blank and placebo samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other excipients. The fig.3 shows that the selected drugs were clearly separated.



(a) (b)

(c) (d)

Fig. 3: Specificity Chromatogram (a) Blank (b) Placebo (c) Standard (d) Sample

Robustness:

Results of the robustness (table no.6). The elution order and resolution for all components were not significantly affected. RSD of peak areas were found to be well within the limit of 2.0%.

System suitability:

The system suitability parameters were found to be within acceptance criteria. Good peak with resolution between two drugs is >1.5, asymmetric factor <2 shows that the three drugs were better separated. Results are tabulated in table 7

Degradation studies:

Results are tabulated in table 8.

Table. 8. Stability Studies for Trifluridine and Tipiracil

Conditions	Drugs	% Degradation	% Of Assay After Degradation
Acid	Trifluridine	5.6	94.4
	Tipiracil	3.5	96.5
Alkali	Trifluridine	2.2	97.8
	Tipiracil	8.2	91.8
Peroxide	Trifluridine	10.9	89.1
	Tipiracil	4.5	95.5
Thermal	Trifluridine	10.7	89.3
	Tipiracil	9.4	93.6

Acid hydrolysis (Fig. 4a)

Upon performance of acid degradation studies 5.6% of Trifluridine and3.5 % of Tipiracil was degraded.

Base hydrolysis (Fig. 4b)

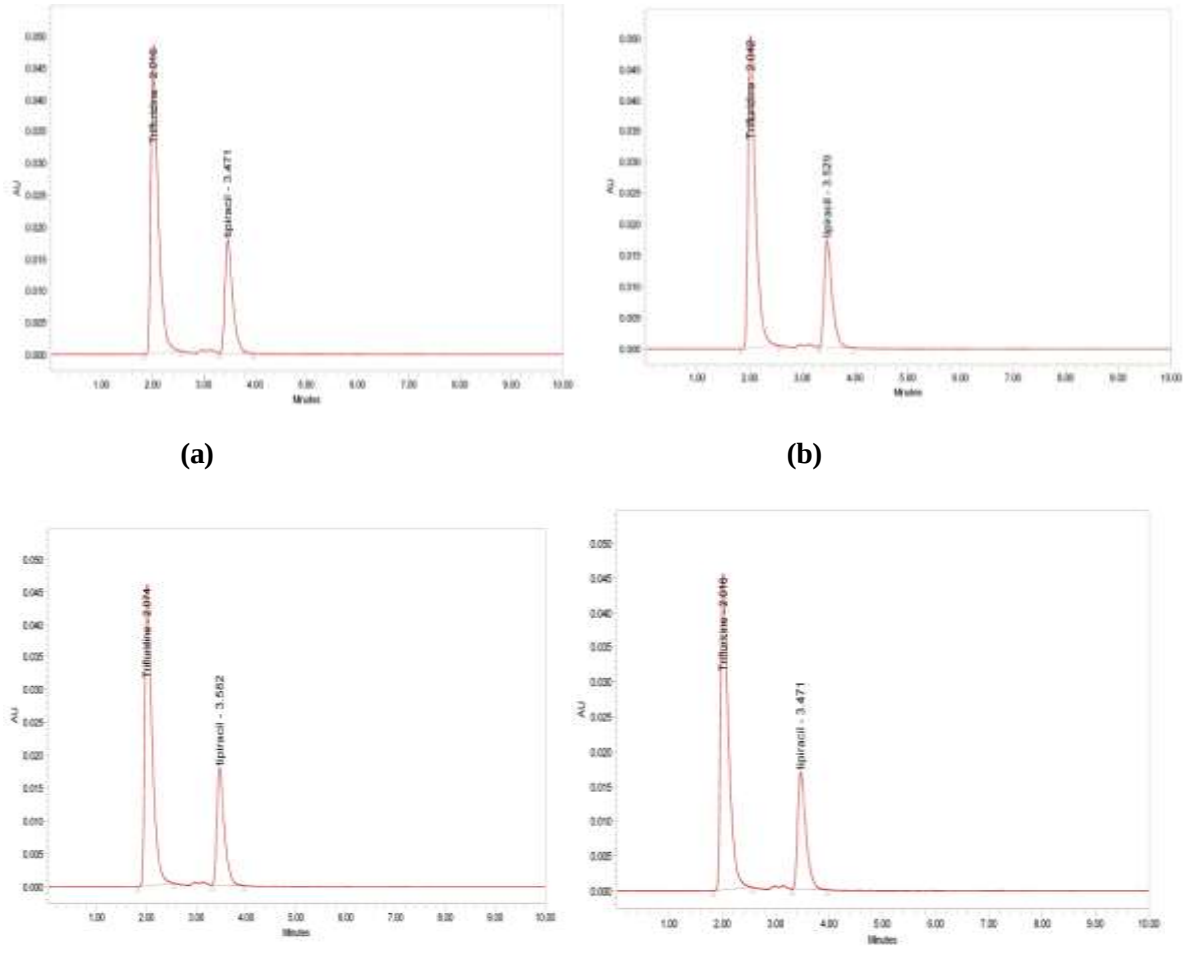
Upon performance of base degradation studies 2.2% of Trifluridine and 8.2% of Tipiracil was degraded.

Peroxide hydrolysis (Fig. 4c)

Upon performance of peroxide degradation studies 10.9 % of Trifluridine and 4.5 % of Tipiracil was degraded.

Thermal degradation (Fig. 4d)

Upon performance of Thermal degradation studies 10.7 % of Trifluridine and 9.4 % of Tipiracil was degraded.



(a) **(b)**

(c) **(d)**

Fig. 4: chromatogram of (a) Acid degradation (b) Alkali degradation (c) Peroxide degradation(d) Thermal degradation

Conclusion

A simple, rapid, accurate and precise stability-indicating RP-HPLC analytical method has been developed and validated for the quantitative analysis of Trifluridine and Tipiracilin bulk drugs and combined

dosage forms. The newly developed RP-HPLC method for separation of different degradation products along with the pure drugs were found to be capable of giving faster retention times while still maintaining good resolution than that achieved with conventional HPLC. This method exhibited an excellent performance in terms of sensitivity and speed. The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies.

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