Research article   **Clinical research**

Reverse Phase-High Performance Liquid Chromatography Method for the Simultaneous Estimation of Trifluridine and Tipiracil in Pure Form and Tablet Dosage Form

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ABSTRACT

A new, simple, precise, rapid, selective and stability reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for the simultaneous quantification of Trifluridine and Tipiracil in pure form and its pharmaceutical dosage form. The method is based on Phenomenex Gemini C18 (4.6×250mm) 5µ column. The separation is achieved using isocratic elution by Methanol: TEA Buffer in the ratio of 65:35% v/v, pumped at flow rate 1.0mL/min and UV detection at 230nm. The column is maintained at 40°C throughout the analysis. The total run time is about 6min. The method is validated for specificity, accuracy, precision and linearity, robustness and ruggedness, system suitability, limit of detection and limit of quantitation as per International conference of harmonization (ICH) Guidelines. The method is accurate and linear for quantification of Trifluridine, Tipiracil between 10 - 50µg/mL and 20 - 100µg/mL respectively. Further, satisfactory results are also established in terms of mean percent- age recovery (100.37% for Trifluridine and 100.34% for Tipiracil, intra-day and inter-day precision (<2%) and robustness. The advantages of this method are good resolution with sharper peaks and sufficient precision. The results indicate that the method is suitable for the routine quality control testing of marketed tablet formulations.

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

INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and food drug administration (FDA) guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation .It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (high performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. The appropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods.

The developed chromatographic methods further validated as per ICH or USFDA guidelines for all the critical parameters. To access the precision and to evaluate the results of analysis the analyst must use statistical methods. These methods include confidence limit, regression analysis to establish calibration curves. In each analysis the critical response parameters must be optimized and recognized if possible.

Pharmaceutical analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches like chemistry, physics and microbiology etc. Pharmaceutical analytical techniques are applied mainly in two areas, quantitative analysis and qualitative analysis, although there are several other applications.

Drugs and pharmaceuticals are chemicals or like substances, which or of organic inorganic or other origin. Whatever may

be the origin, we some property of the medicinal agent to measure them quantitatively or qualitatively.

In recent years, several analytical techniques have been evolved that combine two or more methods into one called "hyphenated" technique e.g. GC/MS, LC/MS etc. The complete analysis of a substance consists of four main steps.

The concept of analytical chemistry lies in the simple, recise and accurate measurements. These determinations require highly sophisticated instruments and methods like mass spectroscopy, gas chromatography, high performance thin layer chromatography, high performance liquid chromatography etc. The HPLC method is sensitive, accurate, precise and desirable for routine estimation of drugs in formulations.

The primary objective of proposed work is
- ➢ To develop new simple, sensitive, accurate and economical analytical method for the simultaneous estimation of Trifluridine and Tipiracil.
- ➢ To validate the proposed method in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of the Trifluridine and Tipiracil dosage form.

MATERIALS AND METHODS

Table 1: Instruments Used

S.No.	Instruments And Glass wares	Model
1	HPLC	WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table 2: Chemicals Used

S.No	Chemical	Brand names
1	Trifluridine	Sura labs
2	Tipiracil	Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT
TRAILS
Preparation of standard solution: Accurately weigh and transfer 10 mg of Trifluridine and Tipiracil working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.3 ml of Trifluridine and 0.6ml of Tipiracil from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure: Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization: Initially the mobile phase tried was methanol: Water, Methanol: Phosphate buffer and ACN: Water with varying proportions. Finally, the mobile phase was optimized to TEA buffer (pH 4.0), Methanol in proportion 65:35 v/v respectively.

Optimization of Column: The method was performed with various C18columns like Symmetry, X terra and ODS column. Phenomenex Gemini C18 (4.6×250mm) 5µ was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS
Instrument used : Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature : 40ºC
Column : Phenomenex Gemini C18 (4.6×250mm) 5µ
Mobile phase : Methanol: TEA Buffer (65:35 v/v)
Flow rate : 1ml/min
Wavelength : 230nm
Injection volume : 10µl
Run time : 6minutes
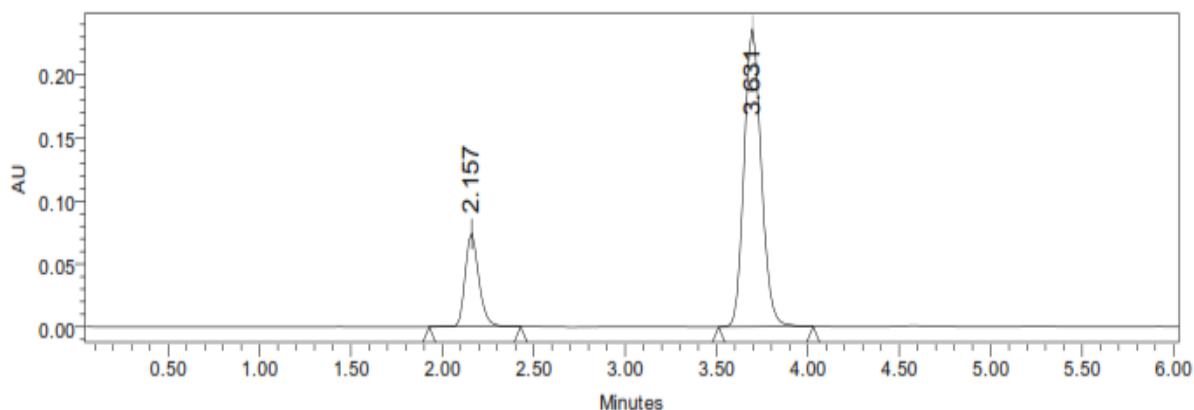
RESULTS AND DISCUSSION
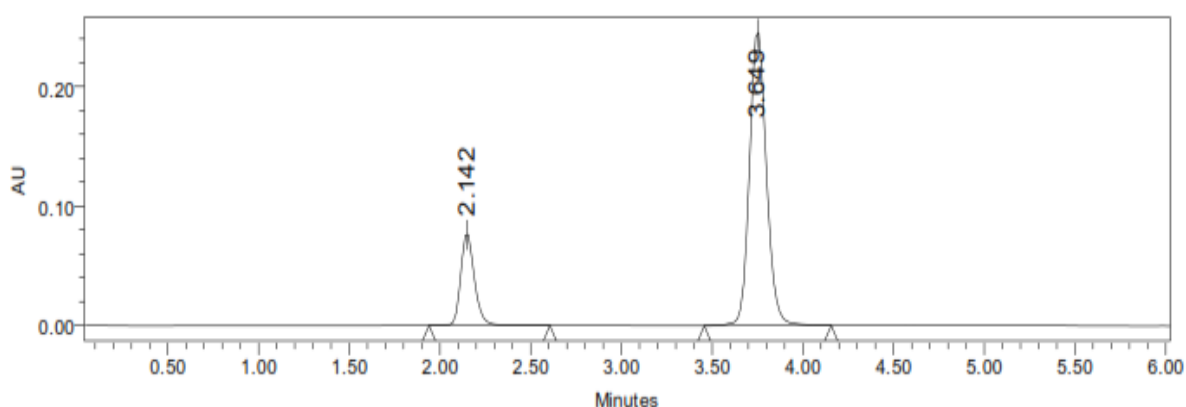

Fig 1: Optimized Chromatogram (Standard)

Fig 2: Optimized Chromatogram (Sample)

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

METHOD VALIDATION
System Suitability

Table 3: Results of system suitability for Trifluridine

S.No.			Area	Height (μV)		
1	Trifluridine	2.152	526856	78569	1.63	5856
2	Trifluridine	2.157	528794	78545	1.63	5874
3	Trifluridine	2.141	526598	78954	1.62	5869
4	Trifluridine	2.133	524875	78224	1.63	5897
5	Trifluridine	2.166	526584	78965	1.62	5829
Mean			526741.4			
Std. Dev.			1392.398			
% RSD			0.264342			

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table 4: Results of system suitability for Tipiracil

S.No			Area	Height			Resolution
1	Tipiracil	3.674	1645985	268542	5869	1.48	10.01
2	Tipiracil	3.631	1648579	267854	5874	1.49	10.01
3	Tipiracil	3.625	1645739	268598	5864	1.48	9.99
4	Tipiracil	3.692	1645285	268745	5826	1.49	10.01

5	Tipiracil	3.629	1648598	268598	5824	1.48	10.02
Mean			1646837				
Std. Dev.			1618.325				
% RSD			0.098269				

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Specificity

Table 5: Peak results for assay standard of Trifluridine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Trifluridine	2.152	526595	78569	1.63	5896	1
2	Trifluridine	2.198	524658	78496	1.63	5879	2
3	Trifluridine	2.179	528476	78459	1.62	5895	3

Table 6: Peak results for assay standard of Tipiracil

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Tipiracil	3.646	1648546	265845	1.48	8012	1
2	Tipiracil	3.604	1648598	265418	1.49	7955	2
3	Tipiracil	3.610	1648574	265365	1.48	7989	3

Table 7: Peak results for Assay sample of Trifluridine

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Trifluridine	2.152	536598	79856	1.64	5969	1
2	Trifluridine	2.150	536589	79265	1.65	5997	2
3	Trifluridine	2.187	534658	79898	1.65	5986	3

Table 8: Peak results for Assay sample of Tipiracil

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Tipiracil	3.646	1658952	278598	1.49	8016	1
2	Tipiracil	3.651	1658954	276984	1.48	8041	2
3	Tipiracil	3.601	1653659	275849	1.49	8079	3

$$\%ASSAY = \frac{Sample\ area}{Standard\ area} \times \frac{Weight\ of\ standard}{Dilution\ of\ standard} \times \frac{Dilution\ of\ sample}{Weight\ of\ sample} \times \frac{Purity}{100} \times \frac{Weight\ of\ tablet}{Label\ claim} \times 100$$

The % purity of Trifluridine and Tipiracil in pharmaceutical dosage form was found to be 99.63%

Linearity
Chromatographic Data For Linearity Study Of Trifluridine

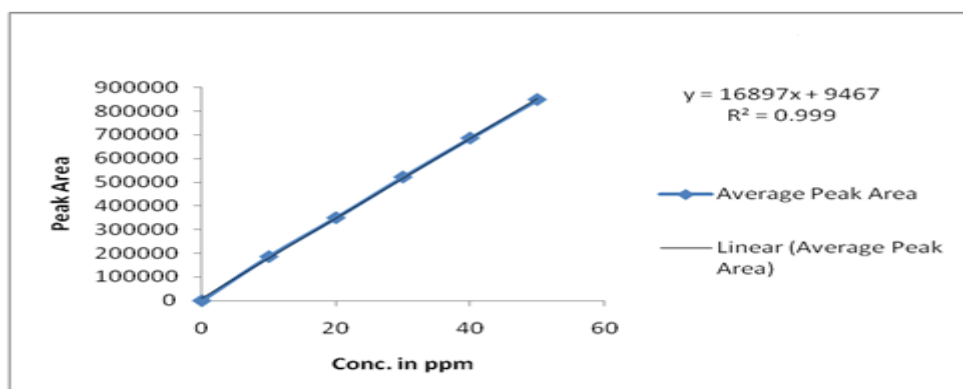| Concentration	Average
10	185689
20	349852
30	521541
40	685986
50	848265

Fig 3: Calibration Curve of Trifluridine

Correlation Coefficient (r) is 0.99, and the intercept is 9467. These   values meet the validation criteria.

Chromatographic Data For Linearity Study Of Tipiracil

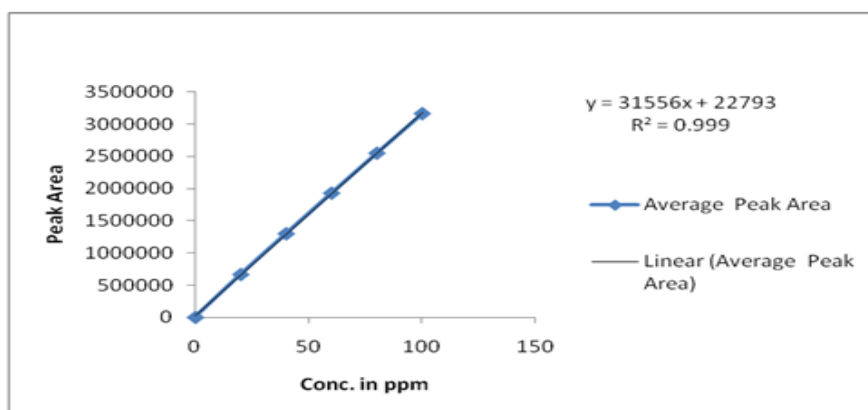| Concentration µg/ml	Average Peak Area
20	665985
40	1298698
60	1927852
80	2548545
100	3162468

Fig-4: Calibration Curve of Tipiracil

Correlation Coefficient (r) is 0.99, and the intercept is 22793. These   values meet the validation criteria.

Precision
REPEATABILITY

Table 9: Results of Repeatability for Trifluridine:

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Trifluridine	2.157	526854	78569	5869	1.62
2	Trifluridine	2.159	523659	78469	5874	1.63
3	Trifluridine	2.186	523856	78525	5896	1.63
4	Trifluridine	2.160	523485	78548	5818	1.62
5	Trifluridine	2.170	523485	78594	5879	1.63
Mean			524267.8			
Std.dev			1453.805			
%RSD			0.277302			

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

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	3.603	1645879	265845	7985	5869
2	Tipiracil	3.608	1648578	265487	7964	5849
3	Tipiracil	3.600	1645985	265982	7915	5879
4	Tipiracil	3.696	1648759	265478	7928	5874
5	Tipiracil	3.629	1648572	265422	7964	5829
Mean			1647555			
Std.dev			1483.603			
%RSD			0.090049			

Intermediate precision

Table 11: Results of Intermediate precision Day 1 for Trifluridine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Trifluridine	2.198	536598	79584	5963	1.64
2	Trifluridine	2.196	536985	79685	5978	1.65
3	Trifluridine	2.160	534587	79654	5947	1.64
4	Trifluridine	2.160	536985	79845	5982	1.65
5	Trifluridine	2.160	536985	79864	5971	1.65
6	Trifluridine	2.186	538568	79685	5968	1.64
Mean			536784.7			
Std. Dev.			1277.909			
% RSD			0.238067			

- %RSD of five different sample solutions should not more than 2.
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Table 12: Results of Intermediate precision Day 1 for Tipiracil

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Tipiracil	3.623	1658254	266598	8036	1.50	10.06
2	Tipiracil	3.611	1659872	266473	8045	1.51	10.04
3	Tipiracil	3.696	1653589	266958	8075	1.50	10.05
4	Tipiracil	3.696	1658458	266451	8049	1.50	10.06
5	Tipiracil	3.696	1653652	266352	8069	1.50	10.05
6	Tipiracil	3.642	1652395	266954	8024	1.51	10.06
Mean			1656037				
Std. Dev.			3175.804				
% RSD			0.191771				

- %RSD of five different sample solutions should not more than 2.

Table 13: Results of Intermediate precision Day 2 for Trifluridine

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Trifluridine	2.198	519689	77859	5749	1.61
2	Trifluridine	2.196	518957	77985	5792	1.60
3	Trifluridine	2.178	519856	77854	5746	1.60
4	Trifluridine	2.142	519857	77869	5749	1.61
5	Trifluridine	2.177	519869	77935	5718	1.61
6	Trifluridine	2.177	519687	77954	5795	1.60
Mean			519652.5			
Std. Dev.			351.0976			
% RSD			0.067564			

- %RSD of five different sample solutions should not more than 2.

Table 14: Results of Intermediate precision Day 2 for Tipiracil

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing	Resolution
1	Tipiracil	3.611	1638598	256985	7968	1.47	9.90

2	Tipiracil	3.623	1637849	257589	7952	1.46	9.91
3	Tipiracil	3.684	1635982	256985	7934	1.46	9.90
4	Tipiracil	3.697	1636598	254613	7986	1.47	9.90
5	Tipiracil	3.684	1635874	258487	7924	1.46	9.91
6	Tipiracil	3.684	1635984	259861	7915	1.47	9.91
Mean			1636814				
Std. Dev.			1145.885				
% RSD			0.070007				

- %RSD of five different sample solutions should not more than 2.

Accuracy

Table 15: The accuracy results for Trifluridine

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	263572	15	15.038	100.253%	
100%	518870.3	30	30.147	100.490%	100.37%
150%	772572.3	45	45.162	100.360%	

- The percentage recovery was found to be within the limit (98-102%).

Table 16: The accuracy results for Tipiracil

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	972935.7	30	30.109	100.363%	
100%	1919319	60	60.100	100.166%	100.34%
150%	2877020	90	90.449	100.498%	

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Limit Of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

TRIFLURIDINE

= 0.9µg/ml

TIPIRACIL

= 1.2µg/ml

QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

TRIFLURIDINE

=2.7µg/ml

TIPIRACIL

Result: =3.6µg/ml

Robustness

Table 17: Results for Robustness Trifluridine

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	526541	2.157	5859	1.62
Less Flow rate of 0.9 mL/min	589564	2.210	5635	1.61
More Flow rate of 1.1 mL/min	515246	2.184	5569	1.64
Less organic phase	502659	2.200	5154	1.63
More Organic phase	526485	2.172	5365	1.62

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Table 18: Results for Robustness Tipiracil

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	1645875	3.643	7965	1.48
Less Flow rate of 0.9 mL/min	1635985	4.498	7856	1.46
More Flow rate of 1.1 mL/min	1624587	3.505	7425	1.43
Less organic phase	1652834	4.504	7621	1.45
More organic phase	1625548	3.512	7582	1.42

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Trifluridine and Tipiracil in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. Trifluridine was found to be soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide; it is very slightly soluble in water, slightly soluble in Acetonitrile and ethanol, sparingly soluble in methanol, practically insoluble in toluene. Tipiracil was found to be very slightly soluble in water (0.9 mg/mL). Tipiracil is soluble in methanol (ca. 60 mg/mL), sparingly soluble in ethanol (ca. 10 mg/mL), very slightly soluble in isopropanol (<1 mg/mL), and very slightly

soluble in acetone. Methanol: TEA Buffer (65:35 v/v) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Trifluridine and Tipiracil in bulk drug and in Pharmaceutical dosage forms.

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