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RP-HPLC APPROACH FOR CONCURRENT QUANTIFICATION OF TRIFLURIDINE AND TIPIRACIL PHARMACEUTICAL AND IN BULK FORMS: DEVELOPMENT AND VALIDATION

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Abstract

The proposed RP-HPLC approach effectively quantifies tipiracil and trifluridine in pharmaceutical dosage forms, offering high sensitivity, linearity, and repeatability. Using a phosphate based buffer pH 4.6 and a carrier liquid of methanol with phosphate buffer solution (70:30 v/v), the method employs a Symmetry C18 column (4.6 x 150mm, 5µm, XTerra) with UV absorbance measurement at 273 nm, the flow capacity of 1.0 ml/min and the run time was carried for 7mins.The retention duration of Tipracil is 2.003min and 5.067minfor Trifluridine. The linearity range is 25-125 µg/ml for both drugs while the regression coefficient lies at ≥ 0.999. The precision of developed method is confirmed by %RSD values under 2%, and recovery rates of 97-102% demonstrate accuracy and minimal excipient interference, making it suitable for routine quality control.

Keywords: Symmetry C18, tipiracil and trifluridine, RP-HPLC.

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Introduction

Trifluridine/Tipiracil is a combination drug used to treat refractory, metastatic colorectal cancer. It combines trifluridine, an anti neoplastic pyrimidine analogue, with tipiracil, which inhibits trifluridine's metabolism [1]. The combination of LONSURF and bevacizumab is designed for patients with metastatic colorectal cancer whose disease has continued to advance despite prior therapies. This approval signifies a significant progress in the treatment landscape for individuals with advanced colorectal cancer who have not responded to other available treatments [2-3]. The creation of robust validation methods is vital for new drugs to confirm their safety, effectiveness, and quality.

Well-designed validation processes are instrumental in detecting and resolving potential problems early in the development phase, thereby minimizing the risk of issues during later stages and ensuring the drug's reliable and consistent performance in clinical applications.

Experimental

Materials:The analytical drugs Trifluridine (FTD) and Tipiracil (TPI) standards were obtained from Standard Laboratories in Hyderabad, India. Lonsurf tablets, each containing 20 mg of FTD and 9 mg of TPI, were acquired from a local market in Hyderabad, India. Acetonitrile, HPLC-grade methanol, potassium dihydrogen orthophosphate, orthophosphoric acid, distilled water, and hydrochloric acid were supplied by Merck and Rankem India.Milli-Q water has been utilized for experimental duration.

Equipment: The experiment was carried out using an HPLC Alliance system assembled with Empower software and a UV detector model UV Win 5. Chromatographic partitioning was performed on a Symmetry C18 column (4.6 x 150 mm, 5 µm, XTerra). Empower 2 software was employed for data capture, and spectral quality was assessed using a diode array detector (DAD).

Standard Preparation:

Procedure followed for Trifluridine Standard Solution: An accurate 10 mg of Trifluridine was placed in a clean and dry 10 ml capacity volumetric flask. Approximately 2 ml of diluent was transferred, and the flask was shaken until the contents were fully dissolved. The final volume was

brought using a diluent to create the stock solution. The prepared solution of Trifluridine is diluted to 100ml aiding diluent, labeled and stored cautiously for further procedure. Procedure followed for Tipiracil Standard Solution: An accurate 10 mg of Tipiracil was placed in a clean and dry 10 ml capacity volumetric flask. About 2 ml of diluent was added, followed by sonication until complete dissolution. Approximately 2 ml of diluent was transferred, and the flask was shaken until the contents were fully dissolved. The final volume was brought using a diluent to create the stock solution. The prepared solution of Tipiracil is diluted to 100ml aiding diluent, labeled and stored cautiously for further procedure.

Procedure followed for standardsolutionand calibration graph4 (Trifluridine and Tipracil):Ten tablets of the marketed formulation containing Trifluridine and Tipiracil were accurately weighed and powdered using mortar and pestle. An equivalent amount of 10 mg of both Trifluridine and Tipiracil was accurately collected and transferred into a clean and dry volumetric flask of 10 ml Capacity. Approximately the diluent of 7 ml was transferred, and the flask was sonicated until complete dissolution. The final volume was then adjusted aiding diluent to obtain the stock solution. From the prepared stock solution, 3 ml was collected and transferred into a 10 mlcapacity volumetric flask and diluted using diluent. This method ensures that standard and sample solutionswere at accurate concentrations and can be used for subsequent analysis by RP-HPLC.

Method Validation

To validate theparticular HPLC method, ICH guidelines (ICHQ1A (R2) and ICHQ1B)5 were followed in terms of linearity, accuracy, precision, robustness, and degradation studies. To determine the assay method precision, five independent test solutions were analyzed. Intermediate precision was assessed by conducting the assay on several days involving different columns of the same dimensions. Method's accuracy was assessed by the addition of known quantities of the analyte to the solution and measuring the recovery rates at levels of 50%, 100%, and 150% for both Tipiracil (90 µg/ml) and Trifluridine (200 µg/ml) in a placebo matrix. Linearity test solutions for Trifluridine and Tipiracil were prepared in the range of 10-50 ppm. The method's robustness was tested by modifying the end experimental conditions and observing the readings. The specific variations included changing the flow velocity by ±0.1 ml/min, adjusting the organic modifier content by ±2%, altering the column temperature by ±5°C, and shifting the eluent phase pH by ±0.1 units.

Results and Discussions

Selection of Detection wavelength: The overlay spectrum was utilized to select the appropriate wavelength for detecting Trifluridine and Tipiracil. The isosbestic point, where the absorbance of both compounds intersects, was chosen as the detection wavelength.

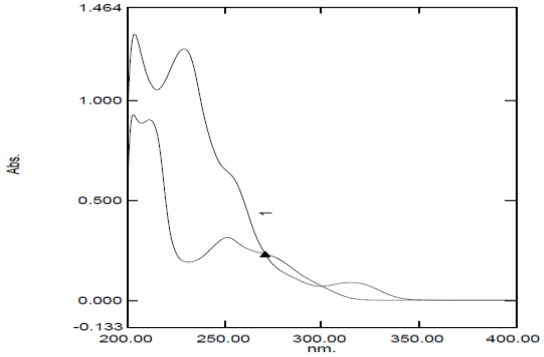


Fig-1 spectrumwavelength for detecting Trifluridine and Tipiracil

Refinement of the chromatographic parameters:

While analysing basic drugs such as Lonsurf, which is a formulation in combination of 20 mg of Trifluridine (FTD) and 9 mg of Tipiracil (TPI), a common issue encountered is peak tailing. This happens because these compounds have strong interactions with the charged ends of the HPLC column filling materials, resulting in considerable peak asymmetry and decreased efficiency. Recent improvements in ultra-pure silica backbones and bonding technologies have greatly reduced the tailing issues related to polar drugs in HPLC6-9.

To optimize the method, various columns were tested, Ultimately the Symmetry C18 column (150 mm × 4.6 mm, 5 µm, XTerra) was found to be effective.. The ideal eluent phase was found to be a solution of 70% methanol and 30% phosphate buffer with a pH of 4.6, adjusted with orthophosphoric acid. To further optimize the chromatographic conditions, various buffers, including phosphate, acetate, and citrate, were tested for eleunt phase preparation. Screening experiments revealed that the combination of 70% methanol and 30% phosphate buffer (pH 4.6) yielded the best peak results. A flow velocity of 1.0 ml/min was maintained to ensure appropriate retention times for the Trifluridine (FTD) and Tipiracil (TPI) peaks.

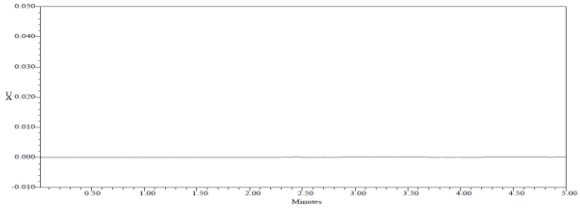


Fig-2 Blank Chromatogram for trifluridine and tipiracil

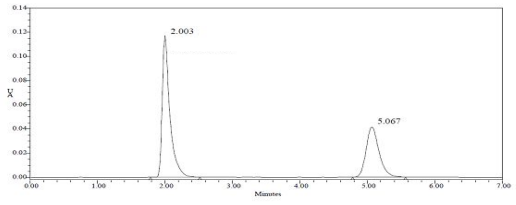


Fig-3 Optimized Chromatogram for trifluridine and tipiracil

Accuracy:

The recovery studies were conducted at three levels: 50%, 100%, and 150% in the determination of accuracy of method. The average recovery in percentage for trifluridine (FTD) and tipiracil (TPI) was found to be 99.66% and 99.76%, respectively.

Table-1 Results depicting Accuracyfor trifluridine and tipiracil

Sample con.	Set no of taken sample	Sample area		Assay		% Recovery	
		FTD	TIP	FTD	TIP	FTD	TIP
50%	1	460064	276931	24.9	25.0	99.8	100
	2	460124	276694	24.6	24.9	99.6	99.6
	3	460216	276891	24.8	24.9	99.8	99.6
	Average Recovery					**99.7%**	**99.7%**
100%	1	932429	554165	49.9	50.0	99.8	100
	2	932654	554879	49.8	49.9	99.6	99.8
	3	932742	565371	49.8	49.9	99.6	99.8
	Average recovery					**99.6%**	**99.8%**
150%	1	1378901	828131	74.8	75.0	99.8	100
	2	1358360	828974	74.9	74.9	99.8	99.8
	3	1368984	828439	74.6	74.8	99.6	99.8
	Average recovery					**99.7%**	**99.8%**

Precision:

Method precision of the HPLC was evaluated by analyzing five individual test solutions. This assessment aimed to determine the consistency and repeatability of the method under the same conditions.

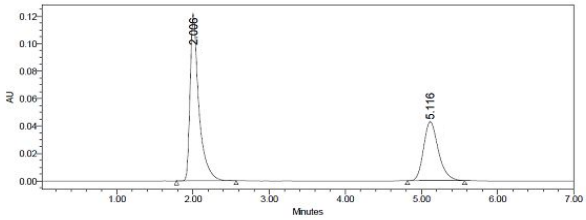


Fig-4 Chromatogram for sample injection -1

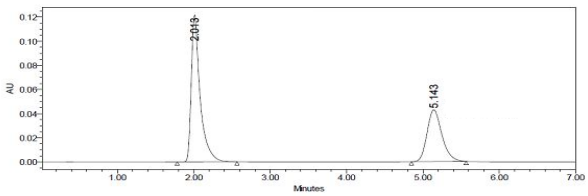


Fig-5 Chromatogram for sample injection-2

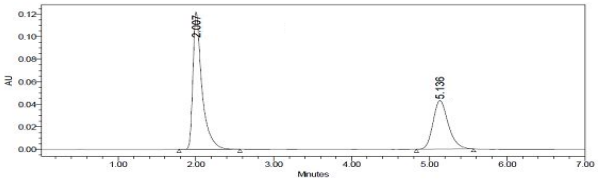


Fig-6 Chromatogram for sample injection-3

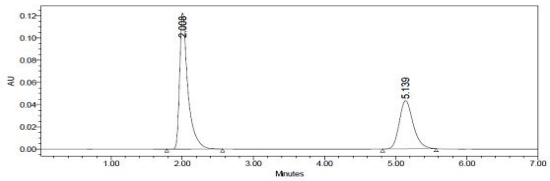


Fig-7 Chromatogram for sample injection-4

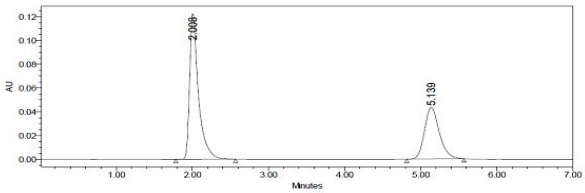


Fig-8 Chromatogram for sample injection-5

Table-2 Results of method precision for trifluridine and tipiracil

S. No	Sample area		Standard area		Percentage purity	
	Trifluridine	Tipiracil	Trifluridine	Tipiracil	Trifluridine	Tipiracil
1	592403	983375	577531	971536	101.36	101.04
2	592352	985049	580381	973007	101.85	101.03
3	592357	982956	577723	975717	102.32	100.54
4	592323	985219	582190	978909	101.44	100.44
5	596525	994145	583378	981422	101.09	101.09
Average					**101.24**	**100.84**
%RSD					**0.46**	**0.304**

Intermediate precession: Intermediate precision was assessed to measure the repeatability of the HPLC method under varied conditions over multiple days.

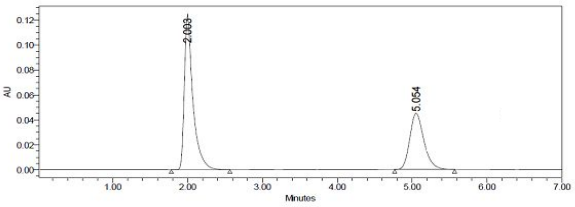


Fig-9 Chromatogram for sample injection-1 DAY-1

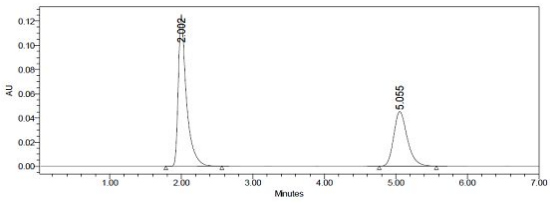


Fig-10 Chromatogram for sample injection-2 DAY-2

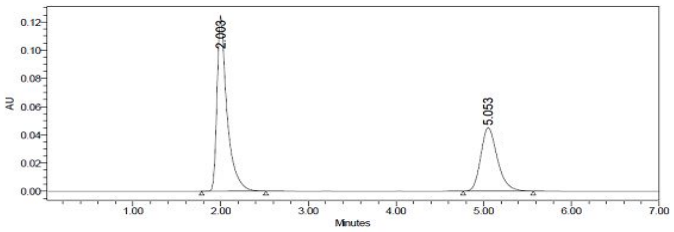


Fig-11 Chromatogram for sample injection-3 DAY-3

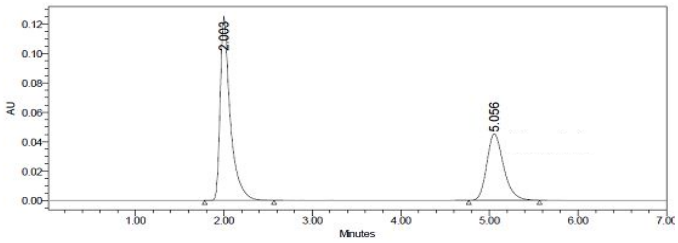


Fig-12 Chromatogram for sample injection-4 DAY-4

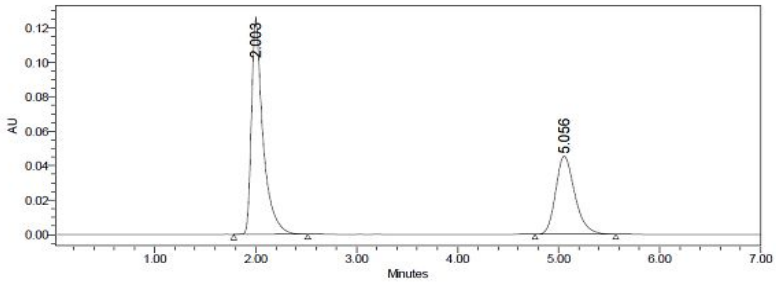


Fig- 13 Chromatogram for sample injection-5 DAY-5

Table- 3 Results of Intermediate precision for trifluridine and tipiracil

S. No	Sample area		Standard area		Percentage purity	
	Trifluridine	Tipiracil	Trifluridine	Tipiracil	Trifluridine	Tipiracil
1	583416	979556	593403	984395	99.12	99.30
2	583657	982467	594352	984039	99.01	99.64
3	584731	979717	593357	983976	99.52	99.36
4	583594	978909	592673	984278	99.61	99.28
5	597649	981432	593671	973915	99.12	100.57
Average					**99.27**	**99.63**
%RSD					**0.27**	**0.54**

Linearity: The linearity range was accessed from 25% to 125% and chromatograms

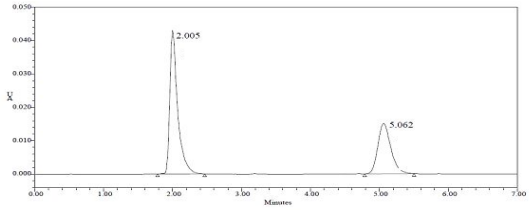


Fig-14 Chromatogram for linearity concentration25%

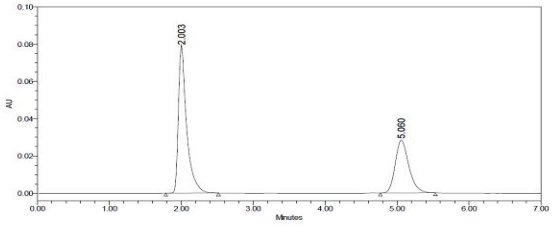


Fig-15 Chromatogram for linearity concentration-50%

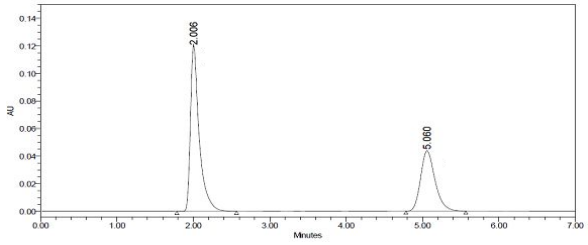


Fig-16 Chromatogram for linearity concentration-75%

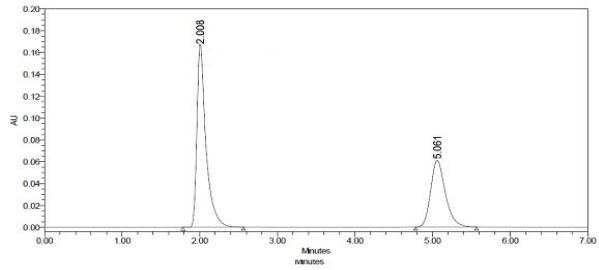


Fig-17 Chromatogram for linearity concentration-100%

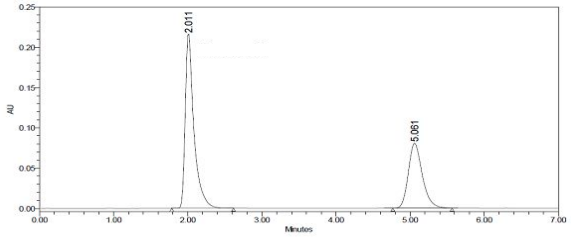
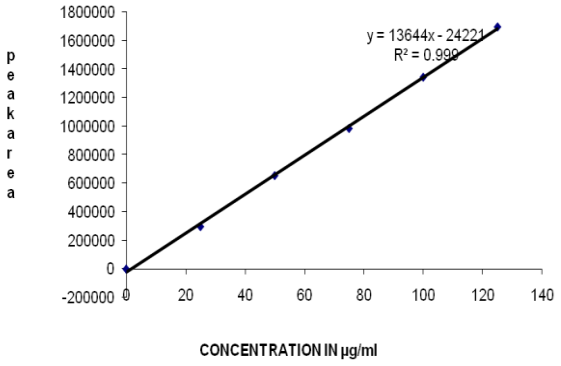


Fig-18 Chromatogram for linearity concentration-125%

Table-4 Linearity Values of the drugs

Con. in (µg/ml)	Peak area of tipiracil	Peak area of trifluridine
25	296809	1798981
50	653820	387871
75	983781	599711
100	1342355	799623
125	1692486	1018914



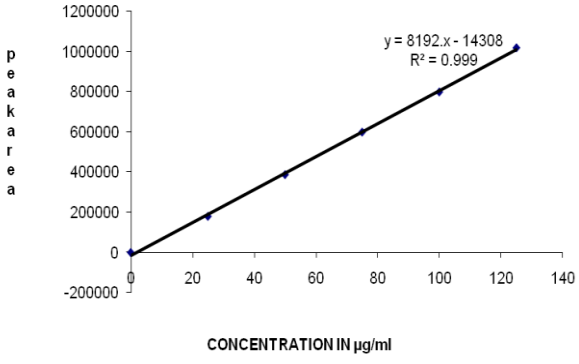


Fig- 19 Calibration graph for TIP at 273 nmFig-20Calibration graph for FTD at 273 nm

Limit of detection for trifluridine and tipiracil: The least possible concentration of the sample was formulated in relation to the baseline noise, and the signal-to-noise ratio was then measured.

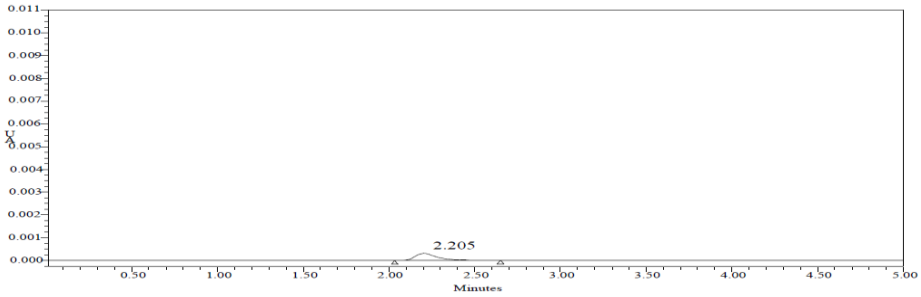


Fig-21 Chromatogram of tipiracil showing LOD

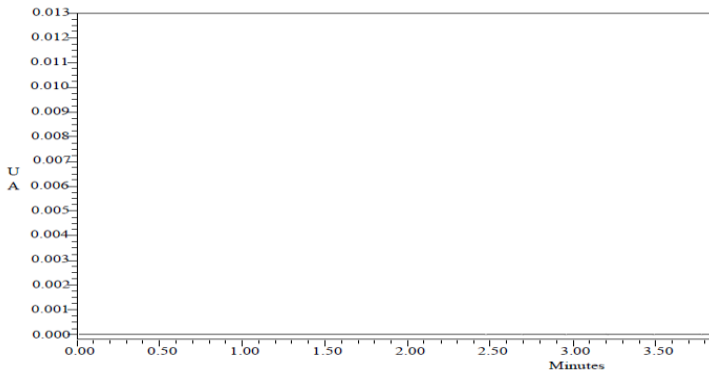


Fig-22 Chromatogram of trifluridine showing LOD

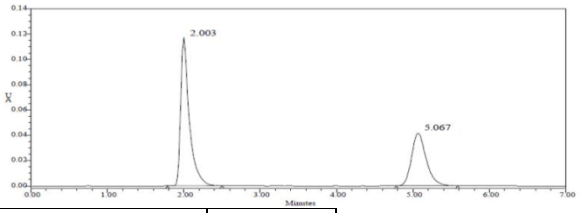


Table-5 LODResults of selected drugs

Drug	Background noise (µV)	Signal recorded (µV)	S/N ratio
Tipracil	56	176	3.14
Trifluridine	56	154	2.75

Limit of quantitation for tipiracil and trifluridine:

possible quantity of the analyte was formulated in relation to the baseline noise, and the signal-to-noise ratio was The least then measured.

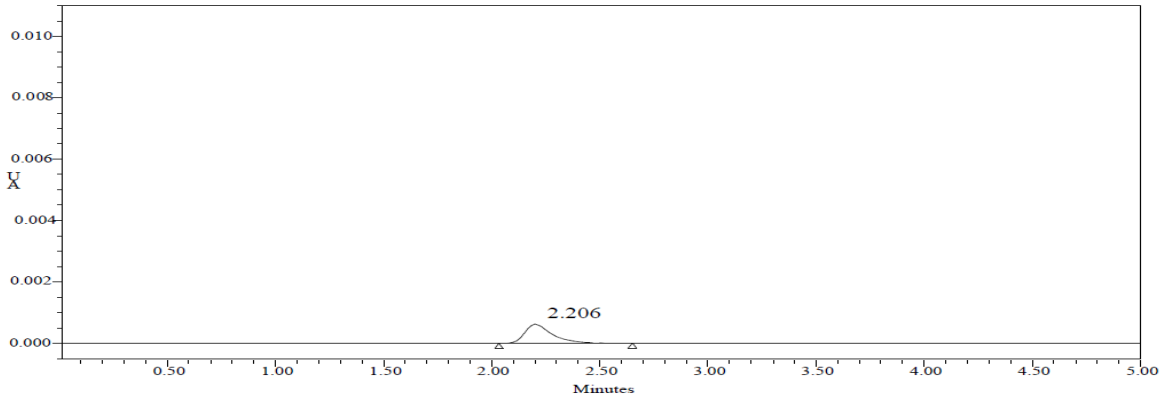


Fig-23 Chromatogram of Tipiracil showing LOQ

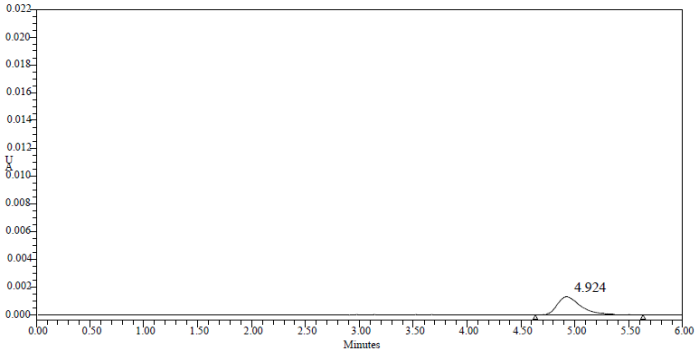


Fig-24 Chromatogram of Trifluridine showing LOQ

Table -6 LOQResults of selected drugs

Drug	Background noise(µV)	Signal recorded(µV)	S/N ratio
Tipracil	56	563	10.05
Trifluridine	56	558	9.96

Robustness:

Standard and sample injections of Tipiracil and Trifluridine were performed with varying chromatography conditions. The parameters such as resolution, tailing factor, asymmetric factor, and plate count remained stable, showing no significant changes.

Variation in flow

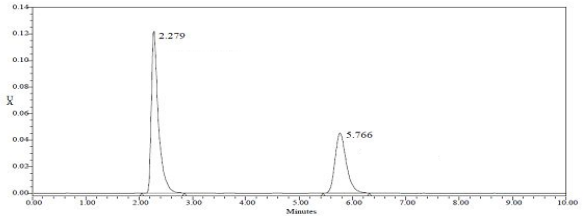


Fig-25 Chromatogram displays reduced flow at 0.7ml/min

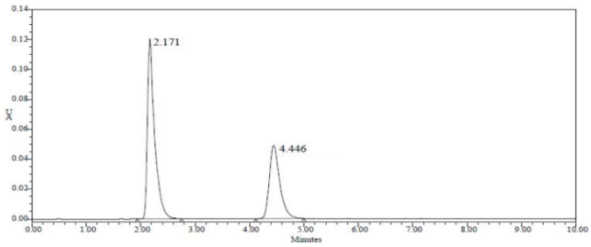


Fig-26 Chromatogram displays enhanced flow of 0.9ml/min

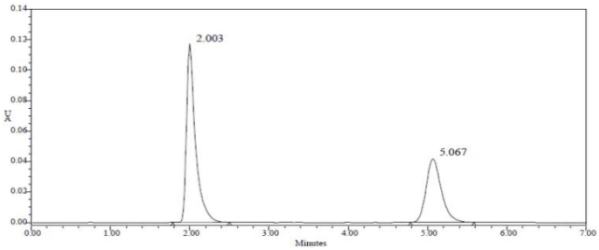


Fig-27 Chromatogram displays lower organic composition

Fig-28 Chromatogram displaysincreased organic composition

Table 7 Results of the impact of flow rate variation

S. No	peak area for Less flow (0.7 ml/min)		peak area for more flow (0.9ml/min)	
	Tipiracil	Trifluridine	Tipiracil	Trifluridine
1	983465	575351	971563	592641
2	985134	580381	973021	592352
3	983467	587724	975674	595471
4	985217	583190	978974	594416
5	994245	584468	984542	583453
Mean	**986406**	**582323**	**976765**	**591767**
%RSD	**0.45**	**0.80**	**0.53**	**0.80**

Table 8Outcomes of the impact of changes in mobile phase composition

S. No	peak area for reduced organic content (70%)		Peak area for increased organic content (90%)	
	Tipracil	Trifluridine	Tipracil	Trifluridine
1	984565	574371	981565	593761
2	986134	585481	983527	592462
3	984268	587627	985489	594491
4	986216	585362	987954	596316
5	995247	585448	994672	587353
Mean	**987396**	**583568**	**986561**	**592787**
%RSD	**0.45**	**0.90**	**0.51**	**0.57**

Conclusion

The quantification of Tipiracil and Trifluridine was performed using RP-HPLC. The eluent phase was optimized to a 70:30 % v/v ratio of methanol and phosphate buffer at pH 4.6. A C18 Symmetry column (150 mm × 4.6 mm, 5 µm, XTerra) was used as the static phase. Detection was recorded with a UV detector at wavelength maximum 273 nm, and chromatographic separation was achieved at a flow rate of 1.0 ml/min. The method demonstrated linearity for both Tipiracil and Trifluridine over the range of 25-125 µg/ml, with a linear regression coefficient of at least 0.999. The method exhibited high precision and accuracy, with %RSD values under 2%. The recovery percentages for Tipiracil and Trifluridine ranged from 98% to 102%. The LOD and LOQ values were within acceptable ranges.

Overall, the developed method is straightforward, precise, and accurate for the analysis of Tipiracil and Trifluridine both in bulk and in its formulations. The high recovery rates suggest that the method effectively avoids

interference from formulation excipients, making it well-suited for routine quality control of these drugs.

Conflict of Interests

The authors declare that there exist no conflicts of interests regarding the publication of this manuscript.

Funding

Nil

Acknowledgement

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Informed Consent

No

Ethical Statement

Not Applicable

Author Contribution

All authors are contributed equally.

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