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METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE ESTIMATION OF TRIFLURIDINE AND TIPIRACIL IN PURE FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

J. Kalyani * , Dr. Gadipally. Sai Kiran, Dr. D. Venkata Ramana.
Department of Pharmaceutical Analysis, Holy Mary Institute Of Technology and Science (College Of Pharmacy), Keesara - Bogaram - Ghatkesar Rd, Kondapur, Telangana, 501301.

Abstract:
A new, simple, Accurate, precise, robust and rugged reverse phase-HPLC method was developed for the simultaneous estimation of the Trifluridine and Tipiracil in pure and pharmaceutical dosage forms. Chromatogram was run through Hypersil C18 (250 mm×4.6 mm, 5µm) particle size. Mobile phase containing Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25%) was pumped through column at a flow rate of 1.0ml/min. Temperature was maintained at Ambient. Optimized wavelength selected was 226 nm. Retention time of Trifluridine and Tipiracil were found to be 1.693min and 3.235min ± 0.02 respectively. The precision %RSD of the Trifluridine and Tipiracil were and found to be 0.435 and 0.039 respectively. %Recovery was obtained as 100.06% and 100.083% for Trifluridine and Tipiracil respectively. Regression equation of Trifluridine is y = 48138x + 5396.0., and y = 71.91x + 42.07 of Tipiracil. The LOD and LOQ values were found to be for the Trifluridine and Tipiracil are 1.27µg/ml, 1.16 µg/ml 3.81µg/ml, 3.48µg/ml and the proposed method was found to be simple, precise, accurate, rapid, economic and reproducible for the estimation of Trifluridine and Tipiracil in pure form and pharmaceutical marketed formulation.

Keywords: *Trifluridine and Tipiracil, HPLC, Method Development, Accuracy, Validation.*

Corresponding author:
J. Kalyani*
Department of Pharmaceutical Analysis,
Holy Mary Institute of Technology and Science (College of Pharmacy),
Keesara - Bogaram - Ghatkesar, Telangana.
Email Id- kalyanijogu2001@gmail.com

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INTRODUCTION:

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. In fact, analytical chemistry is the science of chemical identification and determination of the composition (atomic, molecular) of substances, materials and their chemical structure.

Chemical compounds and metallic ions are the basic building blocks of all biological structures and processes which are the basis of life. Some of these naturally occurring compounds and ions (endogenous species) are present only in very small amounts in specific regions of the body, while others such as peptides, proteins, carbohydrates, lipids and nucleic acids are found in all parts of the body. The main object of analytical chemistry is to develop scientifically substantiated methods that allow the qualitative and quantitative evaluation of materials with certain accuracy. Analytical chemistry derives its principles from various branches of science like chemistry, physics, microbiology, nuclear science and electronics. This method provides information about the relative amount of one or more of these components. [1]

Every country has legislation on bulk drugs and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" (e.g. IP, USP, and BP). Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Every year number of drugs is introduced into the market. Also quality is important in every product or service, but it is vital in medicines as it involves life.

There is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors. Under these conditions standard and analytical procedures for these drugs may not be available in Pharmacopoeias. In instrumental analysis, a physical property of the substance is measured to determine its chemical composition. Pharmaceutical analysis comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. [2]

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production. The decision to release or reject a product is based on one or more type of control action. With the growth of pharmaceutical industry during last several years, there has been rapid progress in the field of pharmaceutical analysis involving complex instrumentation. Providing simple analytical procedure for complex formulation is a matter of most importance. So, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are:

1. The drug or drug combination may not be official in any pharmacopoeias.
2. A proper analytical procedure for the drug may not be available in the literature due to Patent regulations.
3. Analytical methods for a drug in combination with other drugs may not be available.
4. Analytical methods for the quantitation of the drug in biological fluids may not be available.
5. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable. [1, 2]

1.1 DIFFERENT METHODS OF ANALYSIS

The following techniques are available for separation and analysis of components of interest.

Spectral methods

The spectral techniques are used to measure electromagnetic radiation which is either absorbed or emitted by the sample.

E.g. UV-Visible spectroscopy, IR spectroscopy, NMR, ESR spectroscopy, Flame photometry, Fluorimetry.2

Electro analytical methods

Electro analytical methods involved in the measurement of current voltage or resistanceas a property of concentration of the component in solution mixture.

E.g. Potentiometry, Conductometry, Amperometry.[2]

Chromatographic methods

Chromatography is a technique in which chemicals in solutions travel down columns or over surface by means of liquids or gases and are separated from each other due to their molecular characteristics.

E.g. Paper chromatography, thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Gas chromatography (GC). [2]

Miscellaneous Techniques
Mass Spectrometry, Thermal Analysis.

Hyphenated Techniques
GC-MS (Gas Chromatography – Mass Spectrometry), LC-MS (Liquid Chromatography – Mass Spectrometry), ICP-MS (Inductivity Coupled Plasma- Mass Spectrometry), GC-IR (Gas Chromatography – Infrared Spectroscopy), MS-MS (Mass Spectrometry – Mass Spectrometry).
Analytical techniques that are generally used for drug analysis also include biological and microbiological methods, radioactive methods and physical methods etc. are mentioned in Table 1.[2]

List of Chemicals and Reagents used

Ortho-Phosphoric Acid	AR	Finar
Acetonitrile	HPLC	Merck
Methanol	HPLC	Merck
Water	HPLC	Loba Chemi
Potassium dihydrogen orthophosphate	AR	Finar
Trifluridine	NA	Sura Labs
Tipiracil	NA	Sura Labs

METHOD DEVELOPMENT
Instruments:
WATERS HPLC, Model: Alliance 2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. Agilent C8 (4.6 x 150 mm, 5 µm, Make: Waters) column was used for separations.

METHOD DEVELOPMENT:
Optimized Chromatographic parameters:
Mobile phase : Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25)
Auto sample temperature : Ambient
Injection volume : 20µL
Column : Hypersil C18 (250 mm×4.6 mm, 5µm) particle size
Detector wavelength : 226 nm
Flow rate : 1.0ml/min
Run time : 6 minutes
Procedure: a
Inject 20µL of standard, sample into chromatographic system and measure the areas for the Trifluridine and Tipiracil peeks and calculate the % assay by using the formula.

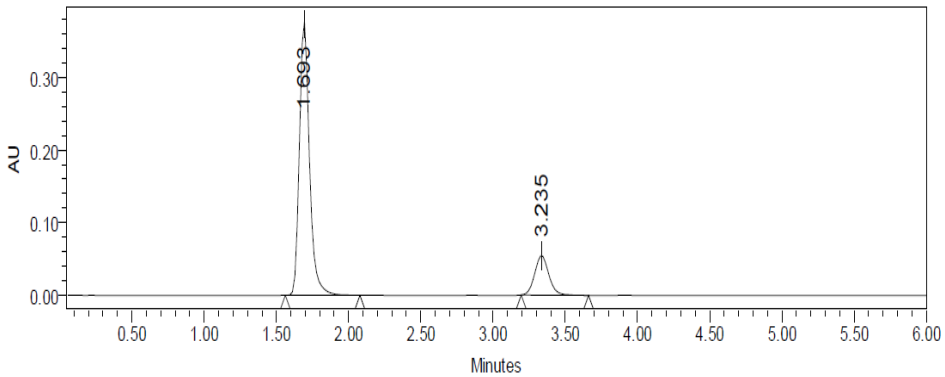

Fig no: 12 Typical Chromatogram for optimized method

Table-: Results of optimized method Chromatogram

S. No.	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1	Trifluridine	1.693	3658985		1.58	5698
2	Tipiracil	3.235	6529	7.28	1.63	7529

Observation: Peeks are well separated all the parameters are within the limits.
PREPARATION OF MOBILE PHASE:
Transfer 1.36086g of Potassium dihydrogen phosphate into 1000ml of beaker and adjust pH 2.80 with orthophosphoric acid (OPA).

Transfer the above solution 750ml and 250ml of methanol is used as mobile phase. They are mixed and sonicated for 20 minutes.

PREPARATION OF THE TRIFLURIDINE AND TIPIRACIL STANDARD AND SAMPLE SOLUTION:

PREPARATION OF STANDARD SOLUTION:

Accurately weigh and transfer 50 mg of Trifluridine and 50 mg of Tipiracil into 50 ml of volumetric flask and add 10ml of water and sonicate 10min (or) shake 5min and make with water.

Further pipette out 0.8ml of Trifluridine and 0.9ml of Tipiracil from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

PREPARATION OF SAMPLE STOCK SOLUTION:

Commercially available six tablets ware weighed and powdered the powdered equivalent to the 585.58 mg of Trifluridine and Tipiracil of active ingredients were transfer into a 50 ml of volumetric flask and add 10ml of methanol and sonicate for 20 min (or) shake 10 min and make up with water.

Further pipette out 0.8ml of Trifluridine and 0.9ml of Tipiracil from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

METHOD VALIDATION

1. SYSTEM SUITABILITY:

Tailing factor for the peaks due to Trifluridine and Tipiracil in standard solution should not be more than 2.0.Theoretical plates for the Trifluridine and Tipiracil peaks in standard solution should not be less than 2500.

Table no: 9 System suitability data of Trifluridine and Tipiracil

parameter	Trifluridine	Tipiracil	Acceptance criteria
Retention time	1.691	3.299	_
Theoretical plates	5698	7529	>2500
Tailing factor	1.58	1.63	<2.00
% RSD	0.02	0.03	<2.00

Table no: 10 Standard Results of Trifluridine

S. no	Sample name	RT	Area	USP plate count	USP tailing
1	Injection 1	1.694	3658986	5698	1.58
2	Injection 2	1.689	3659844	5655	1.59
3	Injection 3	1.692	3659864	5682	1.58
4	Injection 4	1.688	3654875	5674	1.58
5	Injection 5	1.688	3654514	5628	1.59
Avg.			3657617		
SD			2693.969		
% RSD			0.073654		

Table no: 11 Standard Results of Tipiracil

S. no	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection 1	3.244	6598	7598	1.63
2.	Injection 2	3.238	6574	7549	1.64
3.	Injection 3	3.246	6523	7561	1.63
4.	Injection 4	3.265	6539	7592	1.63
5.	Injection 5	3.265	6578	7569	1.64
Avg.			6562.4		
SD			30.59902		
% RSD			0.466278		

RESULT

Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

2. SPECIFICITY:

Solution of standard sample and placebo were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria:

Chromatogram of standard and sample should be identical with near retention time.

Blank interference:

A study to establish the interference of blank was conducted. Solvent was injected into HPLC system as per the test procedure.

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

Table no: 12 Specificity data for Trifluridine and Tipiracil

S. no	Sample name	Trifluridine		Tipiracil	
		Area	Rt	Area	Rt
1	Standard	3658985	1.691	6529	3.299
2	Sample	3785984	1694	6695	3.234
3	Blank	-	-	-	-
4	Placebo	-	-	-	-

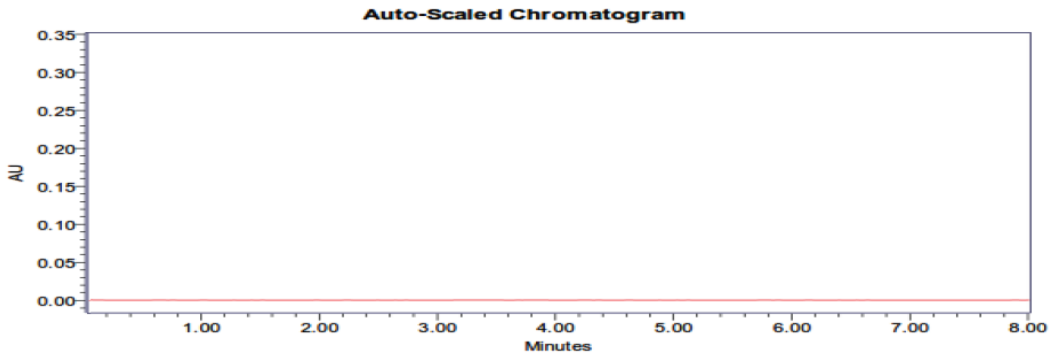

Fig-19: Typical chromatogram of the Blank Solution

ig no: 21 chromatogram representing specificity of standard

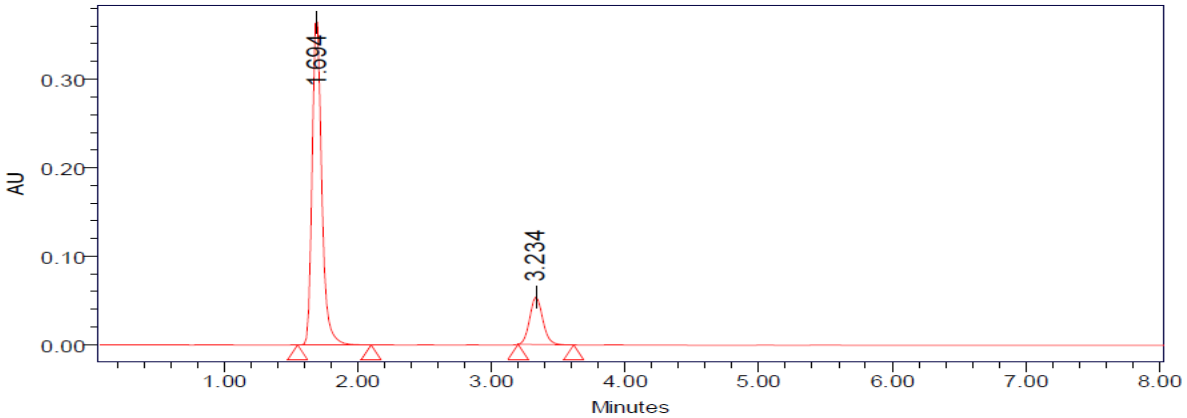

Fig no: 22 chromatogram representing specificity of sample

RESULT

Chromatograms explain that retention time for standard, sample and commercial product of Trifluridine and Tipiracil are same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

3. ACCURACY/RECOVERY:

Recovery study can be performed in the concentration range of 50% to 150% of the target concentration of the test. Minimum 3 concentrations are recommended.

Acceptance criteria:
The average percentage recovery was between 97-103% and relative standard deviation of these recovery concentrations was less than 2%.

Table no: 13 Accuracy data for Trifluridine

S. no	Accuracy level	Injection	Sample area	Rt
1	50%	1	1928492	1.687
		2	1935674	1.691
		3	1927546	1.688
2	100%	1	3859865	1.688
		2	3865143	1.688
		3	3858748	1.688
3	150%	1	5785847	1.686
		2	5786423	1.685
		3	5789658	1.684

Table no: 14 Accuracy (%recovery) results of Trifluridine

S. no	Accuracy Level	Sample name	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	40	39.949	99.872	99.979%
		2	40	40.098	100.245	
		3	40	39.929	99.822	
2	100%	1	80	80.071	100.088	100.124%
		2	80	80.180	100.225	
		3	80	80.048	100.060	
3	150%	1	120	120.080	100.066	100.091%
		2	120	120.092	100.076	
		3	120	120.159	100.132	

Table no: 15 Accuracy data for Tipiracil

S. no	Accuracy level	Sample name	Sample area	Rt
1	50%	1	3287	3.277
		2	3278	3.275
		3	3282	3.266
2	100%	1	6516	3.265
		2	6518	3.265
		3	6529	3.265
3	150%	1	9749	3.268
		2	9758	3.268
		3	9746	3.266

Table no: 16 Accuracy (%recovery) results of Tipiracil

S. no	Accuracy Level	Sample name	µg/ml added	µg/ml found	% Recovery	% Mean
1	50%	1	45	45.124	100.275	100.131%
		2	45	44.999	99.997	
		3	45	45.055	100.122	
2	100%	1	90	90.028	100.031	100.108%
		2	90	90.056	100.062	
		3	90	90.209	100.232	
3	150%	1	135	134.987	99.990	100.010%
		2	135	135.112	100.082	
		3	135	134.945	99.959	

RESULT

Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

% Recovery was 100.00% for Trifluridine and 100.00% for Tipiracil. All the results indicate that the method is highly accurate**.**

4. PRECISION:

Preparation of sample:

Transfer the 802.04mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of methanol and sonicate 20min and makeup with water. Transfer the above solution into 5ml into 25ml volume metric flask dilute to the volume with water.

The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injections.

Acceptance criteria: The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention time are not more than 2.0.

Table no: 17 Precision studies for Trifluridine and Tipiracil

S. no	Intraday precision for Trifluridine			Intraday precision for Tipiracil		
	Peak area	Mean peak area	%RSD	Peak area	Mean peak area	%RSD
1	3658952	3665965	0.435	6598	6547	0.390
2	3659854			6529		
3	3659874			6537		
4	3658748			6538		
5	3698547			6546		
6	3659816			6534		

RESULT

Results of variability were summarized in the above table. The %RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise**.**

5. LINEARITY:

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard versus the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

Acceptance criteria:

Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within ±2.0.

Statistical Evaluation:
A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation coefficient, slope and, y-intercept were calculated.

Table no: 18 Linearity data for Trifluridine

S. no	Concentration (µg/ml)	Rt	Area
1.	40	1.689	1923835
2.	60	1.691	2899874
3.	80	1.692	3868985
4.	100	1.689	4835984
5.	120	1.688	5758747

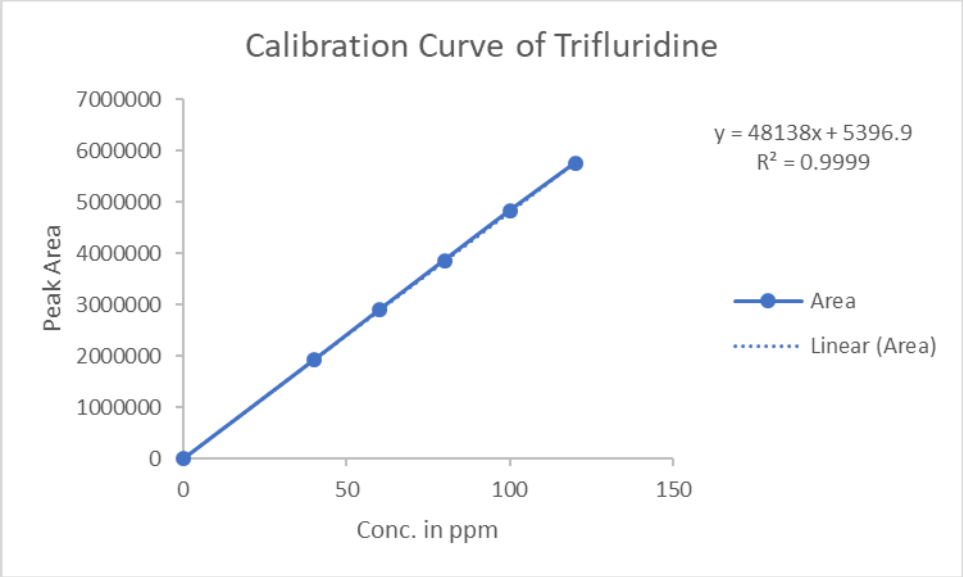

Fig no: 32 Linearity Curve of Trifluridine

Table no: 19 Linearity data for Tipiracil

S. no	Concentration (µg/ml)	Rt	Area
1.	50	3.203	3675
2.	70	3.299	5108
3.	90	3.294	6529
4.	110	3.290	7954
5.	130	3.288	9349

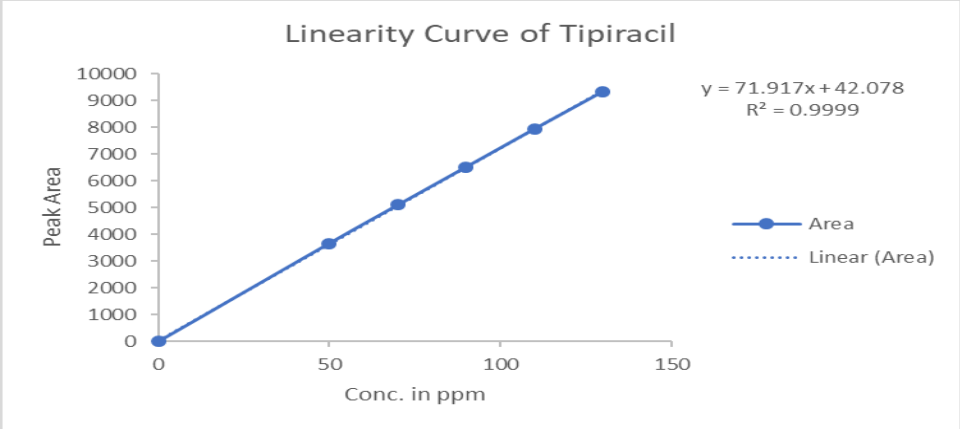

Fig no: 33 Linearity Curve of Tipiracil

RESULT

A linear relationship between peak areas versus concentrations was observed for Trifluridine and Tipiracil in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.999 for Trifluridine and 1 for Tipiracil which prove that the method is linear in the range of 50% to 150%.

6. ROBUSTNESS:

Effect of variation in flow rate:

Prepare the system suitability solution as per the test method and inject into the HPLC system with ± 0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0 ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

Effect of variation in Temperature:

Prepare the system suitability solution as per the test method and injected into the HPLC with ± 5º C of the method temperature. Evaluate the system suitability values as required by the test method for both temperatures.

Table no: 20 Robustness data for Trifluridine

Parameter	Rt	Theoretical plates	Tailing factor
Decreased flow rate (0.8ml/min)	1.868	5854	1.56
Increased flow rate (1.2ml/min)	1.544	5365	1.57
Decreased temperature (20^0c)	1.731	5418	1.53
Increased temperature (30^0c)	1.675	5496	1.54

Table no: 21 Robustness data for Tipiracil

Parameter	Rt	Theoretical plates	Tailing factor
Decreased flow rate (0.8ml/min)	3.621	7598	1.62
Increased flow rate(1.2ml/min)	2.998	7612	1.61
Decreased temperature (20^0c)	6.242	7251	1.64
Increased temperature (30^0c)	2.302	7195	1.61

RESULT

The results of robustness of the present method had shown that changes made in the flow and temperature did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is robust.

7. LIMIT OF DETCTION:

The sensitivity of measurement of Trifluridine and Tipiracil by use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level.

$$LOD = 3.3\ \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curves.

S = mean of slopes of the calibration curves.

The slope S may be estimated from the calibration curve of the analyte.

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

$$LOD = 3.3* \sigma/S$$

Where;
σ = standard deviation of response.
S = slope of calibration curve.

LOD for Trifluridine = 1.27
LOD for Tipiracil = 1.16

8. LIMIT OF QUANTIFICATION:

The sensitivity of measurement of Trifluridine and Tipiracil by the use of proposed method was estimated in terms of limit of quantification (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and the peak area of this blank was calculated at noise level. The LOQ was calculated as ten times the noise value gave the LOQ.

$$LOQ = 10 \sigma / S$$

Where,
σ = standard deviation of intercepts of calibration curves.
S = mean of slopes of the calibration curves.
The slope S may be estimated from the calibration curve of the analyte.
Minimum concentration of standard component in which the peak of the standard gets detected and quantification

$$LOQ = 10*\sigma/S$$

Where;
σ = standard deviation of the response.
S = slope of the calibration curve.

LOQ for Trifluridine = 3.81
LOQ for Tipiracil = 3.48

SUMMARY OF TRIFLURIDINE:

Table no: 22 Summary of validation data for Trifluridine:

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry factor Retention time %RSD	 5698 1.98 1.691 0.03	 Not less than 2500 Not more than 2
2	Specificity a) Blank interference b) Placebo interference	 Specific	 Specific
3	Method precision (%RSD)	0.435	Not more than 2.0%
4	Linearity parameter Slope Intercept Correlation coefficient(r^2)	40-120 µg/ml 48138 5396 0.999	 ≤1
5	Accuracy Mean % recovery	 100.06	 97 - 103%
6	Robustness a) Flow rate variation b) Temperature variation	All the system suitability parameters are within the limits.	

SUMMARY OF TIPIRACIL:

Table no: 23 Summary of validation data for Tipiracil

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Tailing factor Retention time %RSD	 7529 1.63 3.299 0.05	 Not less than 2500 Not more than 2

2	Specificity c) Blank interference d) Placebo interference	Specific	Specific
3	Method precision (%RSD)	0.039	Not more than 2.0%
4	Linearity parameter Slope Intercept Correlation coefficient(r^2)	50-130 µg/ml 71.91 42.07 0.999	≤1
5	Accuracy Mean % recovery	100.083	97 - 103%
6	Robustness c) Flow rate variation d) Temperature variation	All the system suitability parameters are within the limits.	

CONCLUSION:

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative simultaneous 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 freely soluble in methanol and acetone; soluble in water, ethanol, 0.01 M hydrochloric acid, and 0.01 M sodium hydroxide; sparingly soluble in isopropyl alcohol and Acetonitrile; slightly soluble in diethyl ether; and very slightly soluble in isopropyl ether. Tipiracil was found to be soluble in water and it is also soluble in 0.01 M hydrochloric acid and 0.01 M sodium hydroxide; slightly soluble in methanol; very slightly soluble in ethanol; and practically insoluble in Acetonitrile, isopropyl alcohol, acetone, diisopropyl ether, and diethyl ether.
Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25) 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 simultaneous determination of Trifluridine and Tipiracil in bulk drug and in Pharmaceutical dosage forms.

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