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A New Selective Separation method development and Validation of Trifluridine and Tipiracil and its degradents were characterized by LC-MS/MS/QTOF

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Abstract:

A reliable, accurate and simple RP-HPLC method was developed for the simultaneous estimation of Trifuridine and Tipiracil, validated according to ICH guidelines and degradation studies were done by LC-MS/MS/QTOF in tablet dosage form. A column of Symmetry C_{18} (150x4.6mm, 3.5µm) with a flow rate of 1ml/min was used. The combination of 0.1% Tri ethyl amine and Acetonitrile in 70:30 ratio was used as a mobile phase. Trifluridine and Tipiracil peaks were eluted at a retention time of 2.770min, 5.118min respectively. The total run time was 8min. Standard solutions were prepared by dissolving in acetonitrile first and then make up to the mark with mobile phase. The method shows a good linearity in the concentration range of 3-45µg/ml of Trifluridine and 1.3-18.45µg/ml of Tipiracil with correlation coefficient 0.999. This method was validated in terms of specificity, linearity, accuracy, LOD, LOQ, robustness and forced degradation.

Key words: LC-MS/MS, RP-HPLC, Tipiracil, Trifluridine.

1. INTRODUCTION

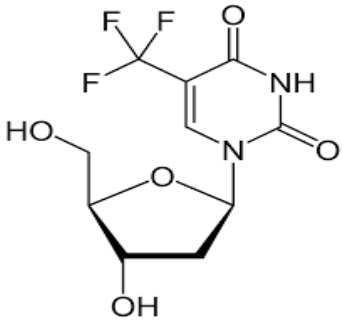
Trifluridine

Trifluridine (also called trifluorothymidine or TFT) is an anti-herpesvirus antiviral drug [1, 2], used primarily on the eye. It was sold under the trade name Viroptic by Glaxo welcome, now merged into GlaxoSmithKline. Trifluridine eye drops are used for the treatment of keratitis [3, 4] and keratoconjunctivitis [5, 6] caused by the herpes [7, 8] simplex virus types 1 and 2, as well as for prevention and treatment of vaccinia [9, 10] virus infections [11] of the eye.

A Cochrane systematic review showed that Trifluridine and acyclovir [12] were a more effective treatment than idoxuridine or vidarabine, significantly increasing the relative number of successfully healed eyes in one to two weeks [13]. For cancer treatment, the combination Trifluridine/tipiracil is used.

Tipiracil

Tipiracil is a drug used in the treatment of cancer [14, 15]. It is approved for use in from of the combination drug Trifluridine/tipiracil for the treatment of unresectable advanced or recurrent colorectal cancer [16, 17, 18]. Tipiracil helps maintain the blood concentration of Trifluridine by inhibiting the enzyme thymidine phosphorylase [19, 20] which metabolizes trifluridine [21]. Adverse effects were not assessed independently of trifluridine, but only in the combination drug.



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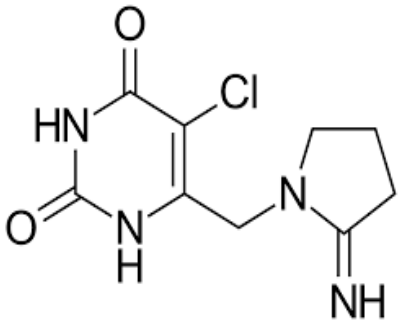

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Fig. No. 1: Structure of (A) Trifluridine and (B) Tipiracil

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Acetonitrile, ortho phosphoric acid (OPA), water and methanol were purchased from Merck (India) Ltd. Worli, Mumbai, India. All API's of Trifluridine, Tipiracil as reference standards were procured from Dr Reddys Laboratories, Hyderabad.

2.2 Instrumentation

Waters alliance e-2695 chromatographic system consisting of quaternary pump, PDA detector-2996 and chromatographic software Empower-2.0 was used.

2.3 LCMS conditions

The chromatographic separations were carried out on a reversed phase Symmetry C18 (150 x 4.6mm i.d.) column with particle size 3.5µm and the column was maintained at an ambient temperature (30°C). HPLC separation was achieved with isocratic elution using 0.1% Tri ethyl amine buffer (pH adjusted to 2.5 with OPA), and acetonitrile as mobile phase in the ratio of 70:30 v/v. The mobile phase was filtered through 0.45µm membrane filter and degassed by using an ultra sonicator before use. The injection volume was 20µl and the mobile phase flow rate was at 1ml/min. A splitter was placed before the ESI source, allowing entry of only 35% of the eluent. The typical operating source conditions for MS scan of ZTP in positive ESI mode were optimized as follows: the fragmentor

voltage was set at 80V; the capillary at 3000V; the skimmer at 60V; nitrogen was used as the drying (300°C; 9 L/min) and nebulising (45psi) gas. Ultra high pure nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions, and are an average 25 scans.

2.4 Preparation of stock and working standards

Preparation of standard solution: Accurately weighed 30mg of Trifluridine and 12.3mg of Tipiracil working standards were transferred into 100ml volumetric flask. Add approximately 70ml of diluents and sonicated for 15min to dissolve the components, after 15min. makeup to the mark with diluents. Further diluted 5ml of the above solution to 50ml volumetric flask and diluted to volume with diluents.

Preparation of Sample solution: 2 tablets were weighed and crush the 2 tablets into powder form, take the sample equivalent to 30mg of Trifluridine and 12.3mg of Tipiracil was transferred into a 100ml volumetric flask and add 70ml of diluents and sonicated for 50mins to dissolve the components and then diluted up to the mark with diluents. Further dilute 5ml of the above solution to 50ml with diluents and it was filtered through 0.45µ nylon syringe filter.

2.5 Method Validation

The analytical method was validated as per ICH Q2 (R_1) guidelines for the parameters like system suitability, specificity, accuracy, precision, linearity, robustness, LOD, LOQ, forced degradation and stability.

2.5.1 System suitability

System suitability parameters like USP plate count, USP tailing and %RSD were measured and found to be within the limits.

2.5.2 Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. It was studied at three different concentration levels (50%, 100% and 150% levels). Minimum three injections were given in each level and percentage of recovery, % RSD was calculated.

2.5.3 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of other components (impurities, degradates or excipients), which may be expected to be present in the sample and standard solution. It was checked by examining the chromatograms of blank samples and samples spiked with Trifluridine and Tipiracil.

2.5.4 Precision

In this method precision was evaluated as system precision, method precision and intermediate precision.

In system precision six replicate standard solutions of Trifluridine and Tipiracil were analyzed and %RSD was calculated.

In method precision six preparations with sample were injected and %RSD, % recovery were calculated. The intraday and inter-day precision study were conducted for both Trifluridine and Tipiracil.

2.5.5 Linearity and range

Linearity was conducted by preparing different standard solutions of Trifluridine and Tipiracil at different concentration levels. The standard solutions were prepared in the concentration range of 3-45µg/ml of Trifluridine and 1.3-18.45µg/ml of Tipiracil. Each concentration was injected into the HPLC system and record the areas obtained. Plot a graph between area taken on Y-axis and concentration on X-axis.

2.5.6 LOD and LOQ

LOD was measured by diluting the standard solution of Trifluridine and Tipiracil and determining the concentration was response of sample peaks are three times the noise peak. LOQ was measured by diluting the standard solution of Trifluridine and Tipiracil and determining the concentration was response of sample peaks are ten times the noise peak.

2.5.7 Stress degradation studies

Forced degradation studies were used to evaluate the specificity of the method. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and peak purity of the principle peaks shall pass. Forced degradation studies were performed by different types of stress conditions to obtain the degradation of about 20%.

2.5.8 Robustness

In robustness the method was determined by making slight changes in the flow rate ±20%, organic phase ±10%, wave length by ±5nm.

2.5.9 Stability

Analytical solution was prepared and injected into the HPLC system at time intervals between 0 hours to 24 hours at 6hours intervals depending on the instrument utilization and sequence of injection.

3. Results And Discussion

3.1 Method development and optimization

The most suitable isocratic condition to resolve Trifluridine and Tipiracil with Symmetry C_{18} column, after the chromatographic conditions were optimized for specificity, resolution and retention time was a mobile phase consisting of 0.1% Tri ethyl amine and Acetonitrile in the ratio of 70:30. When a higher percentage of mobile phase was used, the resultant chromatogram had an increase either in back ground noise or peaks indicating the tailing effect. Thus based on the above mentioned parameters, Trifluridine and Tipiracil were eluted at a retention time of 2.770 min and 5.118 min respectively. Table 1 depicts the chromatographic parameters applied for the method.

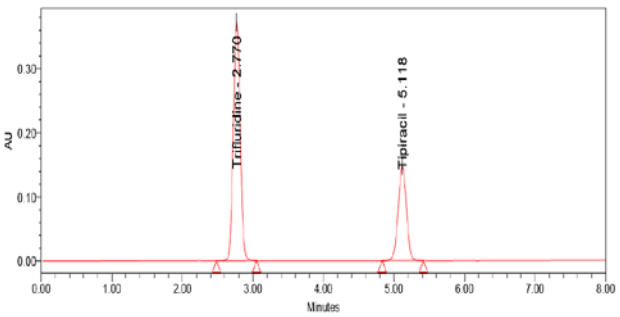


Fig. No. 2: Representative chromatogram of Trifluridine and Tipiracil

Table 1: HPLC isocratic method for Trifluridine and Tipiracil

S. No.	Parameter	Method Conditions
1	Column	Symmetry C_{18} 150x4.6mm, 3.5µ
2	Flow rate	1 ml/min
3	Wave length	261nm
4	Injection Volume	10µl
5	Run time	8 min
6	Mobile phase	0.1% TEA: ACN 70:30

3.2 Method Validation

The method was validated according to the validation of analytical procedures provided in the ICH guidelines and draft guidance for the industry, analytical procedures and method validation.

System suitability

The standard solution was introduced into the HPLC system and found that system suitability parameters are within the limits. The %RSD was calculated to standard peak areas. The system precision results were tabulated in table 2 and the chromatogram of standard was exhibited in the figure 3.

Table 2: Results of system precision

S. No	System suitability parameter	Acceptance criteria	Drug Name	
			Trifluridine	Tipiracil
1	% RSD	NMT 2.0	0.18	0.26
2	USP Tailing	NMT 2.0	1.05	0.99
3	USP Plate count	NLT 3000	3661	9074

Specificity

In specificity samples were prepared by adding equivalent weight of API and placebo with test concentration and then injected into HPLC system. Interference was not found for the chromatograms of placebo solution, empty cell solution and impurities at the retention time of Trifluridine and Tipiracil.

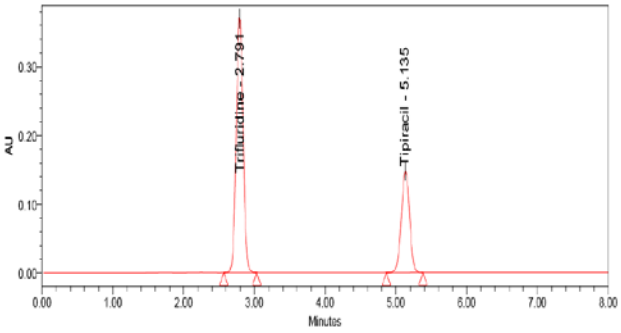


Fig. No. 3: Chromatogram of standard

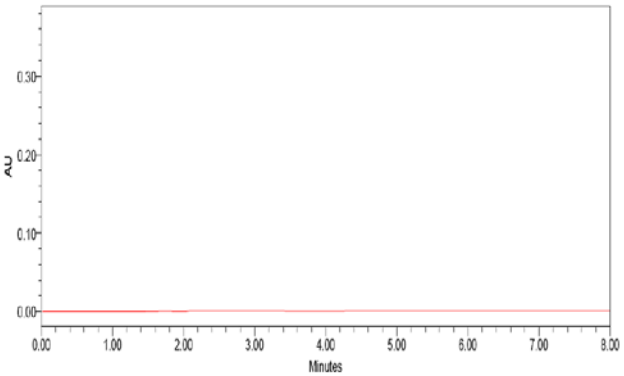


Fig. No. 4: Chromatogram of placebo

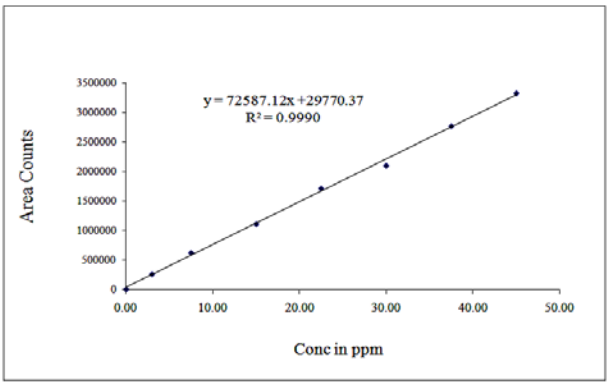
Linearity

Trufluridine linearity concentration was prepared in the range of 3-45µg/ml. The regression equation was found to be Y= 72587.12x+29770.37 and correlation coefficient was 0.999.

Tipiracil linearity concentration was prepared in the range of 1.3-18.45µg/ml. The regression equation was found to be Y=49842.22x+1881.50 and correlation coefficient was 0.9992

Table 3: Results of linearity

S. No.	Trifluridine		Tipiracil	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	3.00	256890	1.23	62716
Linearity-2	7.50	621356	3.08	171818
Linearity-3	15.00	1105500	6.15	308822
Linearity-4	22.50	1711265	9.23	453374
Linearity-5	30.00	2096456	12.30	595546
Linearity-6	37.50	2771404	15.38	760828
Linearity-7	45.00	3325525	18.45	941815
Slope		72587.12		49842.22
Intercept		29770.37		1881.50
CC		0.99904		0.99925



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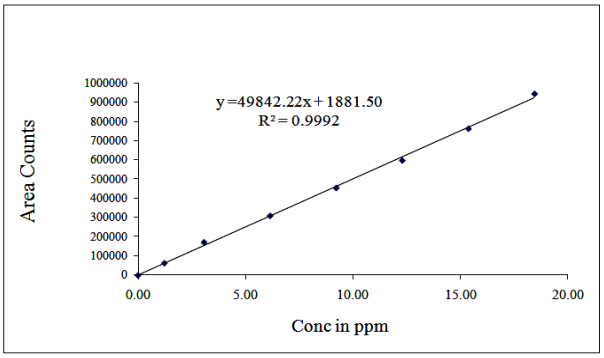

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Fig. No. 5: Linearity plot of (A) Trifluridine and (B) Tipiracil

Robustness

In robustness there is a small deviation in flow rate (±0.2ml) and organic solvent (±10%) in their chromatic condition and observed that there is no significant change in %RSD.

Table 4: Results of robustness

S.No	Parameter name	% RSD for purity	
		Trifluridine	Tipiracil
1	Flow (0.8ml/min)	0.40	0.06
2	Flow (1.2ml/min)	0.87	0.15
3	Organic solvent (+10%) (33:67)	0.64	0.45
4	Organic solvent (-10%) (27:73)	0.23	0.47

Stability

Stability of Trifluridine and Tipiracil was determined in sample solution was studying initial to 24hr at different time intervals at room temperature. The results indicates that there is no significant deviation of purity.

Precision

Precision of the method was established by injecting test preparation and tested through the complete analytical procedure from sample preparation to the final result.

Table 5: Results of stability

S.No	Stability	Purity of Trifluridine in RT	Purity of Trifluridine in 2-8°C	Purity of Tipiracil in RT	Purity of Tipiracil in 2-8°C
1	Initial	100	100	99.3	99.3
2	6Hr	99.3	99.1	98.8	98.8
3	12Hr	99	98.9	97.9	98
4	18Hr	97.9	98.8	97.5	97.6
5	24Hr	97	97.3	96.9	97.3

Table 6: Results of method precision

Analyte	Std Conc.	%RSD
Trifluridine	30	0.4
Tipiracil	12.29	0.23

Intermediate Precision

Six replicates of a sample solution was anlaysed on a different day, different analyst and different RSD values.

Table 7: Results of Intermediate precision

Analyte	Std. Conc.	%RSD
Trifluridine	30	0.29
Tipiracil	12.29	0.77

Limit of Detection and Limit of Quantification (LOD & LOQ)

The LOD concentrations of Trifluridine and Tipiracil were 0.03µg/ml and 0.013µg/ml and LOQ concentrations of Trifluridine and Tipiracil was 0.3µg/ml and 0.13µg/ml respectively.

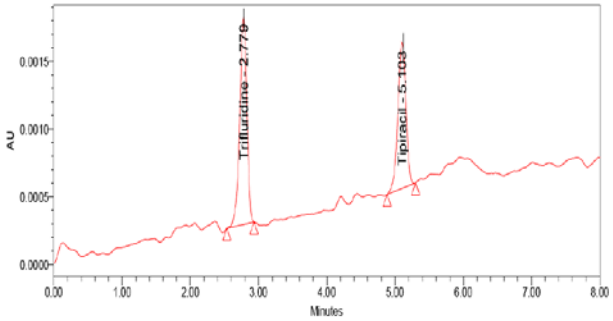

Fig. No. 6: Chromatogram of LOD

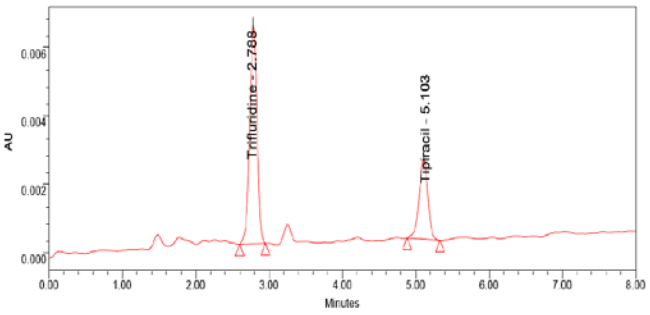

Fig. No. 7: Chromatogram of LOQ

Accuracy

Accuracy was determined by recovery studies which were carried out in three different concentration levels (50%, 100% and 150%). APIs with concentration 15, 30 and 45µg/ml of Trifluridine and 6.15, 12.3 and 18.45µg/ml were prepared. The percentage recovery values were found to be in the range of 98-102%.

Table 8: Results of Accuracy of Trifluridine

S. No.	% Level	% Recovery	Ave % Recovery
1	50	100.5	100.2
2		100.3	
3		100.0	
4	100	99.7	99.8
5		99.9	
6		99.8	
7	150	99.6	99.9
8		99.9	
9		100.3	

Table 9: Results of Accuracy of Tipiracil

S. No.	% Level	% Recovery	Ave %Recovery
1	50	99.5	98.9
2		98.7	
3		98.5	
4	100	99.6	99.8
5		99.8	
6		100.1	
7	150	99.1	99.4
8		99.3	
9		99.8	

Degradation effects and its characterization

Trifluridine and Tipiracil sample was subjected into various forced degradation conditions to effect partial degradation of the drug. Forced degradation studies were performed to show
the method is suitable for degraded products. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities. This degradation samples are characterized by using LCMS.

Acid Degradation

Initially Trifluridine and Tipiracil were studied in 0.1N HCl there is no degradent peaks are formed. When the strength of acid was increased to 1N HCl and heated at 60°C for 30mins, 15% of Trifluridine and 15% of Tipiracil degradation was observed and two degradation products was formed on acid hydrolysis which are eluted with RT 1.758 and 2.118 min.

Alkali Degradation

Initially Trifluridine and Tipiracil was studied in 0.1N NaOH there is no degradent peaks are formed. When the strength of alkali was increase to 1N NaOH and heated at 60°C for 30mins, 12% of Trifluridine and 12% of Tipiracil degradation was observed and two degradation products was formed on alkali hydrolysis which are eluted with RT 1.769 and 2.251 min.

Oxidation

Initially Trifluridine and Tipiracil were studied in 10% peroxide there is no degradent peaks are formed. Then the strength of peroxide was increased to 30% and reflux for 3hrs 17% of Trifluridine and 20% of Tipiracil degradation was observed and one degradent was formed and their RT 1.765 min.

Reduction

First trial of Trifluridine and Tipiracil were studied in 10% sodium bi sulphate solution was used to study the reduction degradation there was no degradent was formed. After that the above solution refluxed for 3hrs 16% of Trifluridine and 15% of Tipiracil was observed and three degradents are formed their RTs are 1.767, 2.113 and 2.251min.

Thermal Degradation

For the first trial of thermal degradation sample was exposed at 105°C for 3hrs and the exposed sample was analyzed there was no degradent peaks are formed. After that the above solution was refluxed for 3hrs 15% of Trifluridine and 12% of Tipiracil degradation was observed and no degradation peaks are formed.

Photolytic Degradation

For the first trial of photolytic degradation sample was exposed in UV light for 6Hrs and the exposed sample was analyzed there was no degradent peaks are formed. After that the exposed sample was refluxed for 3hrs 11% of Trifluridine and 15% of Tipiracil degradation was observed and one degradent peak was formed and their RT 2.019.

Table 10: Forced degradation results of Trifluridine

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	99.9	-	0.145	5.028
Acid Degradation	88.56	11.34	0.142	5.032
Alkali Degradation	85.31	14.59	0.138	5.124
Peroxide Degradation	82.98	16.92	0.156	5.236
Reduction Degradation	83.69	16.21	0.168	5.039
Thermal Degradation	86.35	13.55	0.172	5.051
Photolytic Degradation	88.49	11.41	0.189	5.044

Table 11: Forced degradation results of Tipiracil

Degradation Condition	% of Purity	% of Degradation	Purity Angle	Purity Threshold
Unstressed Degradation	100.0	-	0.087	5.008
Acid Degradation	85.33	14.67	0.089	5.005
Alkali Degradation	87.68	12.32	0.094	5.028
Peroxide Degradation	80.49	19.51	0.064	5.124
Reduction Degradation	85.33	14.67	1.102	5.051
Thermal Degradation	88.18	11.82	1.025	5.041
Photolytic Degradation	85.16	14.84	1.001	5.024

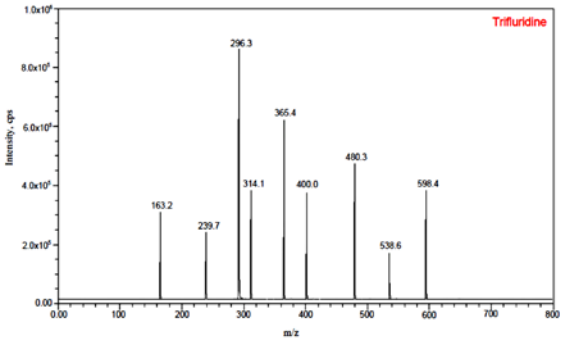

Fig. No. 8: MS Spectra of Trifluridine

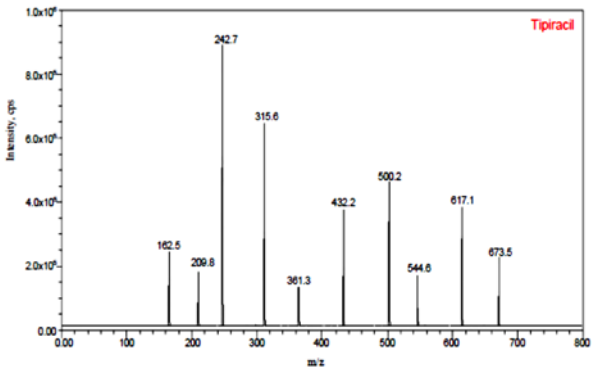

Fig. No. 9: MS Spectra of Tipiracil

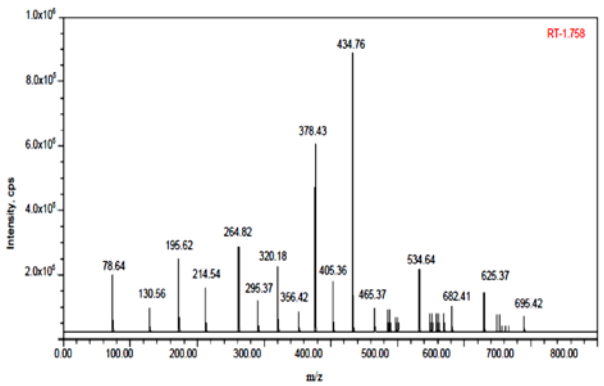

Fig. No. 10: MS Spectra of RT 1.76

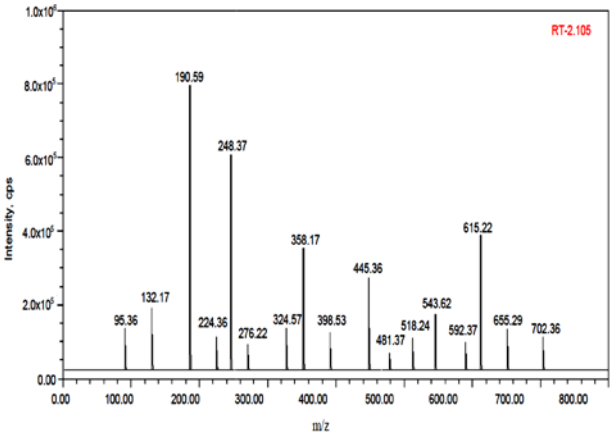

Fig. No. 11: MS Spectra of RT 2.11

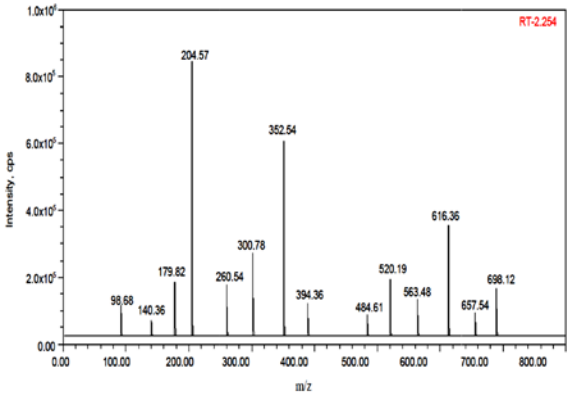

Fig. No. 12: MS Spectra of RT 2.25

4. DISCUSSION

In the present study, to separate trifluridine and tipiracil we use reverse phase HPLC, Symmetry C_{18} column, 0.1% Tri ethyl amine and acetonitrile (70:30) as mobile phase. The reliability, accuracy and precision within the ICH and FDA limits for the method validation of analytical samples. In addition, analysis of the marketed preparation of trifluridine and tipiracil with the validated assay methods showed that the drug contents eluted with no interfering peaks generated by the excipients in the marketed products. Results for robustness and the method were found to remain unaffected by changing the method parameters.

5. CONCLUSION

A validated LC-MS/MS method for stability indicating assay of trifluridine and tipiracil was developed. The degradation behaviour of the drug was investigated under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions. The drug was found to best able in basic, neutral conditions and unstable in oxidative conditions.

An isocratic RP-HPLC method for the determination of trifluridine and tipiracil was developed and is precise and reliable. The regression line equation is capable of reliably predicting the drug concentration in the range of 3-45 µg/ml of trifluridine and 1.3-18.45 µg/ml of tipiracil respectively, from the peak area obtained. The method was successfully validated and allowed the reliable, sensitive,

robust and specific detection of trifluridine and tipiracil in a common marketed preparation.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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