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Research Article

Development and Validation of a New Stability Indicating RP-HPLC Method for the Determination of Trifluridine

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Abstract

Trifluridine is an anti-viral drug used for treatment and prevention of vaccinia viral infections on eye. Trifluridine is used to treat herpes viral infections as well as the inflammation of eye. The present study describes the development and validation of RP-HPLC method for the estimation of Trifluridine in ophthalmic preparations using Shimadzu Model CBM-20A/20 Alite HPLC system consisting of PDA (Photo diode array) detector. A mixture of Formic acid: Methanol (45:55, v/v) was used as mobile phase with flow rate 1.0 mL/min. The proposed method was developed using C_{18} Agilent column (250 mm × 4.60 mm, 5 µm) on an isocratic mode at room temperature with UV detection at 259 nm. Trifluridine obeys Beer-Lambert's law over the concentration range 0.5-120 µg/mL with linear regression equation y = 40447x - 793.8 (R^2 = 0.9997). The method was validated as per ICH guidelines and showed excellent precision, robustness, accuracy, linearity, specificity and the system suitability parameters were within the acceptable criteria. The limit of detection (LOD) and limit of quantification (LOQ) values were found to be 0.1541 µg/mL and 0.4691 µg/mL respectively. Forced degradation studies were performed by exposing Trifluridine to stress conditions such as acidic hydrolysis, alkaline hydrolysis, thermal degradation and oxidation and the degradant products were separated successfully from the API.

Keywords: Trifluridine; Limit of Quantification (LOQ); Limit of Detection (LOD)

Introduction

Trifluridine (Figure 1) is a fluorinated pyrimidine nucleoside which is structurally related to idoxuridine and it is also known as trifluoro thymidine. Trifluridine is an Anti-viral drug and is a white crystalline powder with molecular formula $C_{10}H_{11}F_3N_2O_5$ and molar mass 296.21 g/mol. Chemically it is 1-[(2R,4S,5R)-4-hydroxy-5 (hydroxyl methyl) oxolan-2-yl]-5-(tri Fluoro-methyl) pyrimidine-2,4-dione [1]. Trifluridine is used for treatment and prevention of vaccinia viral infections on eye [2]. Trifluridine acts by inhibiting the viral DNA synthesis which gets incorporated into viral DNA replication and forms defective proteins and causes increased mutation rate [3,4]. Trifluridine is available in combination with Tipiracil in pharmaceutical dosage forms with different brand names in India.

Literature survey reveals that liquid chromatographic methods [5-12] were developed for the simultaneous determination of Trifluridine and Tipiracil in pharmaceutical dosage forms as well as in human plasma [13]. Asha., *et al*. developed a LC-MS/MS method [14] for the estimation of Trifluridine and Tipiracil in tablet dosage forms. Spandana Yasaswini., *et al*. developed a spectrophotometric

[15] method for the estimation of Trifluridine using methanol, water, phosphate buffer (pH 7.0, 2.0, 4.0), NaOH and borate buffer (pH 9.0). Mohammad., *et al*. developed a LC-MS/MS technique [16] for the estimation of Trifluridine in human plasma in presence of the internal standard, *β*-thymidine. In this technique, mobile phase mixture consisting of acetonitrile, methanol and 5 mM ammonium formate (45:40:15, v/v) was allowed to pass through Phenomenex-RP-C18 column with flow rate 0.8 mL/min and the linearity was observed as 5- 2000 ng/mL. Spandana Yasaswini., *et al*. developed a stability indicating RP-UFLC method [17] for the quantification of Trifluridine using mobile phase mixture, Acetonitrile: water (50:50, v/v) with flow rate 0.8 mL/min (UV detection at 261 nm) and the linearity in this method was observed as 1-100 µg/mL. In the present study the authors have developed a simple stability indicating RP-HPLC method for the estimation of Trifluridine in ophthalmic solutions and the method was validated as per ICH guidelines (ICH: International Council for Harmonisation).

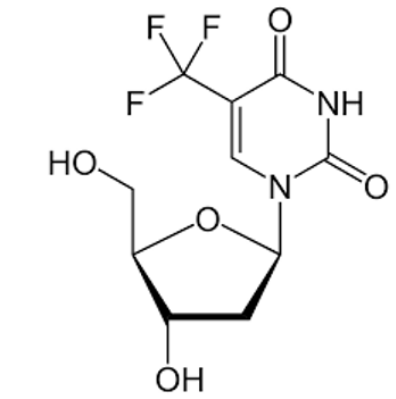


Figure 1: Chemical structure of Trifluridine.

Materials and Methods

Shimadzu Model CBM-20A/20 Alite HPLC system with PDA detector and C_{18} Agilent column (250 mm × 4.60 mm, 5µm particle size) was used for the present study which is maintained at room temperature. Trifluridine is a new drug and is available as ophthalmic solution only. Trifluridine is available with brand name VIROPTIC from Sandoz Falcon Pharma and Pfizer Laboratories Div Pfizer Inc. as 1% ophthalmic solution.

100 mg of Trifluridine (API) was weighed accurately and transferred into a 25 mL volumetric flask and dissolved in methanol (HPLC grade) (1000 µg/mL). The solution was sonicated and dilutions were made with the mobile phase (Formic acid: Methanol; 45:55 v/v) and all the solutions were filtered before use.

Method validation [18]

Linearity, precision, accuracy and robustness

Trifluridine solutions (0.5-120 µg/mL) were prepared from the stock solution on dilution with mobile phase and each solution was injected three times into the HPLC system and the peak area was noted from each chromatogram and finally the mean peak area was calculated. A standard graph was drawn by plotting the concentration of Trifluridine solutions on the x-axis and the respective mean peak area values on the y-axis. Intraday and interday precision studies were performed on the same day and on three successive days at different concentration levels and the statistical parameters were computed. Accuracy study was performed at three levels (50, 100 and 150%) using standard addition method and the percentage recovery was calculated. Robustness study was performed by incorporating small incremental changes in the optimised chromatographic conditions. In all the validation parameters the % RSD was calculated.

Assay of ophthalmic solution (1%)

The ophthalmic solution of Trifluridine extracted with methanol and make up to volume in a volumetric flask to get a formulation stock solution of 1000 µg/mL. This solution was sonicated for 30 min and filtered through membrane filter. 20µL of this formulation solution was injected in to the HPLC system and the peak area was noted from the chromatogram obtained.

Forced degradation studies [19]

Various stress conditions such as alkaline hydrolysis, acidic hydrolysis, heat and oxidation were applied to Trifluridine drug solution. Acidic hydrolysis was performed by treating Trifluridine drug solution with 1 mL of 0.1N HCl, heated at 70ºC for 40 minutes on thermostat, cooled and then neutralized with 1 mL 0.1N sodium hydroxide solution. The resultant mixture was diluted as per the requirement with the mobile phase and 20 µL of this solution was injected into the HPLC system to get the representative chromatogram. Alkaline hydrolysis was performed by treating Trifluridine drug solution with 1 mL of 0.1N NaOH, heated at 70ºC for 40 minutes on thermostat, cooled and then neutralized

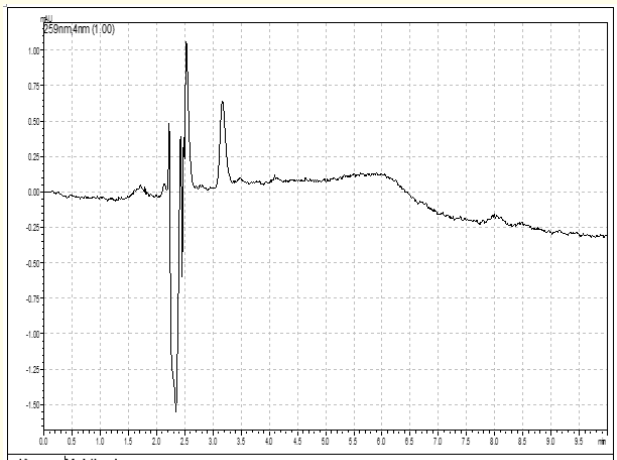
with 1 mL 0.1N hydrochloric acid solution. The resultant mixture was diluted as per the requirement with the mobile phase and 20 µL of this solution was injected in to the HPLC system to get the representative chromatogram. Oxidative degradation was performed by treating Trifluridine drug solution with 1 mL of H_2O_2, heated at 70ºC for 40 minutes on thermostat, cooled and then diluted with the mobile phase as per the requirement and 20 µL of this solution was injected in to the HPLC system to get the representative chromatogram. The drug solution with heated at 80ºC for 30 minutes on a water bath and then cooled. The solution is made up to the volume with mobile phase for required concentration. 20 µL of the solution was injected into the HPLC system. Thermal degradation was performed by heating Trifluridine drug solution at 70ºC for 40 minutes on thermostat, cooled and then diluted with the mobile phase as per the requirement and 20 µL of this solution was injected into the HPLC system to get the representative chromatogram.

Results and Discussion

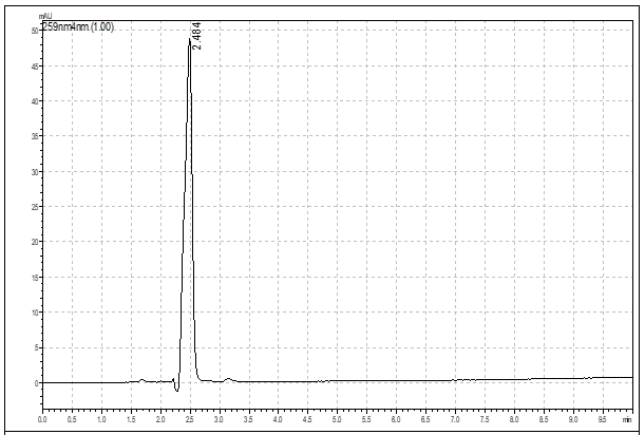
Trifluridine is an anti-viral drug and a stability indicating RP-HPLC method has been developed for the estimation of Trifluridine in ophthalmic solutions. The HPLC system was operated with mobile phase mixture consisting of formic acid: Methanol (45: 55 v/v) and flow rate 1.0 mL/min (UV detection at 259 nm). A detailed review of the previous literature on Trifluridine was given in table 1. Most of the literature was available for the simultaneous estimation of Trifluridine and Tipiracil and very few methods were developed for Trifluridine alone. Some of the important parameters such as the analytical technique, the mobile phase, its composition, linearity were compared with the previously published methods. Initially a baseline was developed by injecting mobile phase alone (Figure 2A) and later Trifluridine (10 µg/mL) was injected using the mobile phase composition, Formic acid: Methanol in 30: 70 v/v ratio with flow rate 0.8 mL/min by which the drug peak was eluted at 2.484 min and tailing factor 0.867 (Figure 2B) but fails to be symmetrical and also the theoretical plates (1317.43) were less than 2000. Therefore, the mobile phase composition, Formic acid: Methanol was modified as 45: 55 v/v ratio along with flow rate (1.0 mL/min) due to which Trifluridine was eluted at 2.326 min with theoretical plates, 3447.045 and tailing factor 1.358 (Figure 2C).

Method	Reagent / Mobile phase	Linearity (µg/mL)	Reference
Spectrophotometry	Methanol Water Phosphate buffer (pH 7.0) Phosphate buffer (pH 2.0) Phosphate buffer (pH 4.0) NaOH Borate buffer (pH 9.0)	10-80 10-80 10-80 10-100 10-100 10-100 10-100	15
LC-MS/MS (Human plasma) (Internal standard: β-thymidine)	Acetonitrile: Methanol: 5 mM Ammonium formate (45:40:15, v/v)	0.005-2.0	16
RP-UFLC	Acetonitrile: water (50:50, v/v)	1-100	17
RP-HPLC	Formic acid: Methanol (45:55, v/v)	0.5-120	Present method

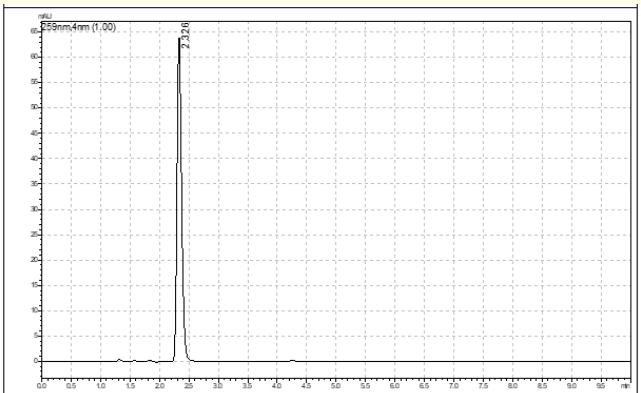
Table 1: Literature survey of Trifluridine.



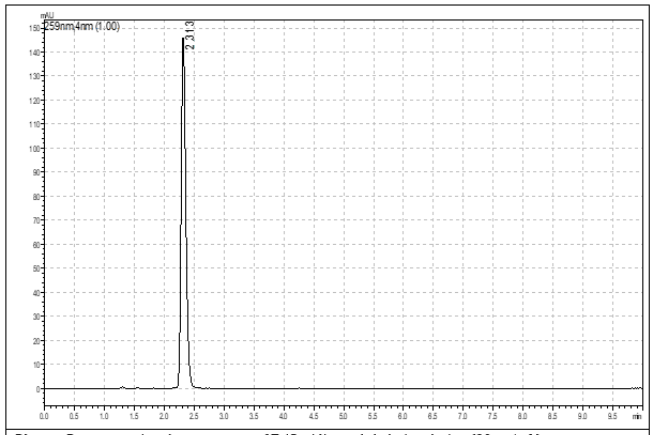
A) Mobile phase



B) Representative chromatogram of Trifluridine (10 µg/mL) (Trial run)
Formic acid: Methanol (30:70, v/v); Flow rate 0.8 mL/min; Rt 2.484 min;
Theoretical plates: 1317.43; Tailing factor: 0.867



C) Representative chromatogram of Trifluridine API (10 µg/mL)
(Optimized conditions) Formic acid: Methanol (45:55, v/v); Flow rate 1.0 mL/min;
Rt 2.326 min; Theoretical plates: 3447.045; Tailing factor: 1.358



D) Representative chromatogram of Trifluridine ophthalmic solution (20 µg/mL)
Formic acid: Methanol (45:55, v/v); Flow rate 1.0 mL/min; Rt 2.313 min;
Theoretical plates: 3774.039; Tailing factor: 1.214

Figure 2: Typical chromatographs Trifluridine.

Linearity, precision, accuracy and robustness

Trifluridine follows Beer-Lambert's law over the concentration range 0.5-120 µg/mL (Table 2) with linear regression equation y = 40447x – 793.8 (R^2 = 0.9997) (Figure 3). The LOD and LOQ values were found to be 0.1541 µg/mL and 0.4691 µg/mL respectively. The method is precise as the % RSD in intraday precision (0.0134-0.1941), interday precision (0.0142-0.9137) (Table 3) was found to be less than 2.0%. In the accuracy study the % RSD was found to be 0.491-0.923 (<2) (Table 4) and that of robustness study was 0.71-1.38 (Table 5) (<2.0) indicating that the method is accurate and robust.

Table 2: Linearity.

Conc. (µg/mL)	*Mean peak area	% RSD
0	0	0
0.5	21343	0.61
1	41254	0.28
2	80152	0.37
5	202108	0.22
10	399257	0.57
20	805012	0.39
40	1611592	0.49
50	2017013	0.28
80	3212659	0.72
100	4032018	0.81
120	47989542	0.43

*Mean of three replicates.

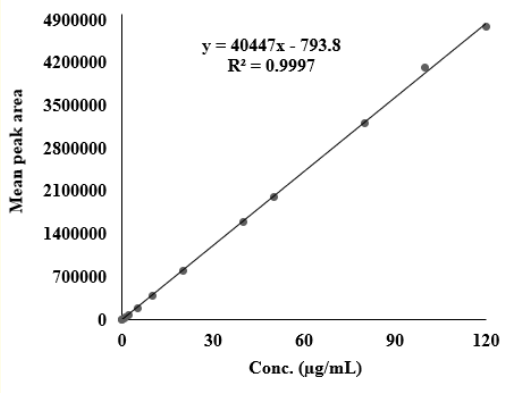


Figure 3: Calibration curve of Trifluridine.

Table 3: Precision study.

Intraday precision study				
Conc. (µg/mL)	**Mean peak area**	**Statistical analysis *Mean peak area ± SD (% RSD)**		
10	399257	399138.33 ± 774.8454 (0.1941)		
10	398311			
10	399847			
20	805012	804666.67 ± 314.4047 (0.0391)		
20	804397			
20	804591			
40	1611592	1611827.33 ± 216.4840 (0.0134)		
40	1612018			
40	1611872			
Interday precision study				
Conc. (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Statistical analysis *Mean peak area ± SD (% RSD)**
10	399257	401298	406394	402316.33 ± 3675.8597 (0.9137)
20	805012	804918	805146	805025.33 ± 114.5833 (0.0142)
40	1611592	1621687	1612942	1615407.00 ± 5480.3672 (0.3393)

*Mean of three replicates

Table 4: Accuracy study.

Spiked conc. (µg/mL)	Formulation (µg/mL)	Total conc. (µg/mL)	*Conc. obtained (µg/mL) ± SD (%RSD)	% Recovery
5 (50 %)	10	15	14.81 ± 0.0727 (0.491)	98.73
10 (100 %)	10	20	19.91 ± 0.1047 (0.526)	99.55
15 (150 %)	10	25	24.89 ± 0.2297 (0.923)	99.56

*Mean of three replicates.

Table 5: Robustness study of Trifluridine (20 µg/mL).

Parameter	Condition	*Mean peak area	*Mean peak area ± SD (RSD)
Flow rate (± 0.1mL/min)	0.9	812459	803740.00 ± 1112.7161 (1.38)
	1.0	805012	
	1.1	796243	
Detection wavelength (± 2 nm)	261	802947	804571.33 ± 8116.9763 (1.01)
	259	805012	
	257	803261	
Mobile phase composition Formic acid: Methanol (± 5 %)	40: 60	798542	804470.33 ± 5676.9145 (0.71)
	45: 55	805012	
	50: 50	809857	

*Mean of three replicates.

Assay of trifluridine ophthalmic solution

Trifluridine 1% ophthalmic solution was procured from two different manufacturers and the proposed RP-HPLC method was applied with the optimized chromatographic conditions. The percentage of purity of Trifluridine was found to be 99.67-99.83 (Table 6). The representative chromatogram of Trifluridine was shown in Figure 2D.

Table 6: Assay of Trifluridine ophthalmic solution.

S. No.	Brand name	Label claim (1 %)	*Observed amount (gm/100 mL)	% Recovery*
1	Brand I	1 gm/100 mL	0.9983	99.83
2	Brand II	1 gm/100 mL	0.9967	99.67

*Mean of three replicates.

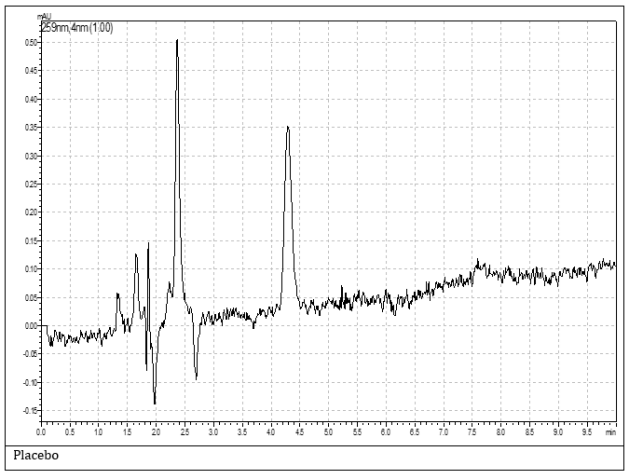
Forced degradation studies

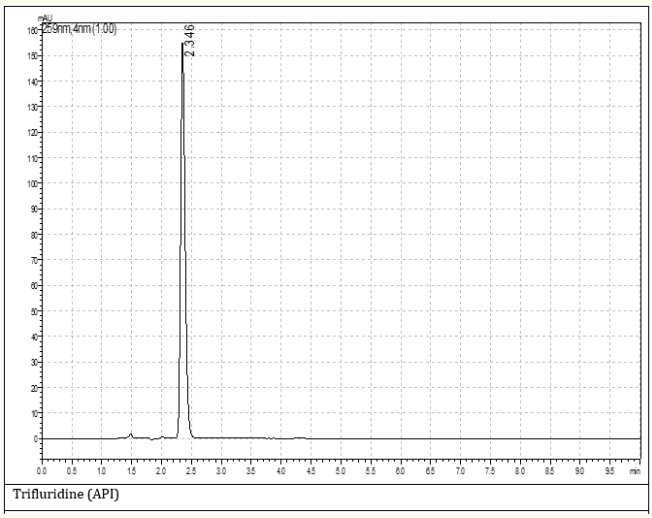
Forced degradation studies were performed to Trifluridine (20 µg/mL) with the optimized conditions. The standard Trifluridine was eluted at 2.346 min with theoretical plates 3809.898 and tailing factor 1.384. Less than 3% of degradation was reported during acidic hydrolysis (Rt 2.313 min), alkaline hydrolysis (Rt 2.337 min), oxidative degradation (Rt 2.326 min) and thermal degradation (Rt 2.315 min). During alkaline hydrolysis and oxidative degradation extra peaks were observed at 1.997 min and 1.822 min respectively. In all the degradations the theoretical plates were greater than 2000 and the tailing factor was less than 1.5 indicating that the system suitability parameters were within the acceptable criteria (Table 7). The typical chromatograms of Trifluridine observed during the forced degradation studies were shown in figure 4.

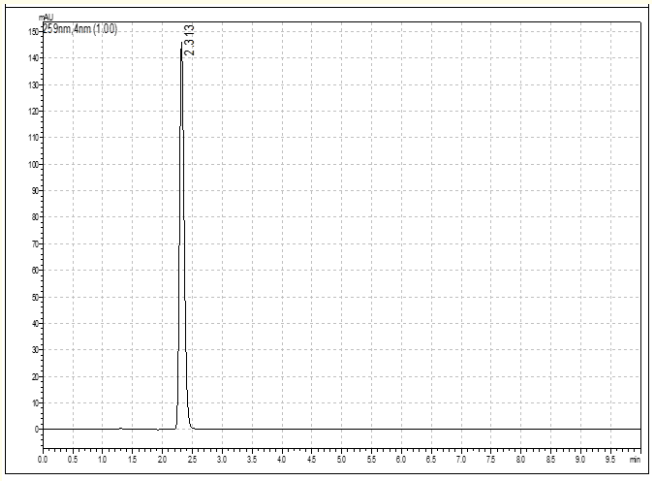
Table 7: Stress degradation studies of Trifluridine.

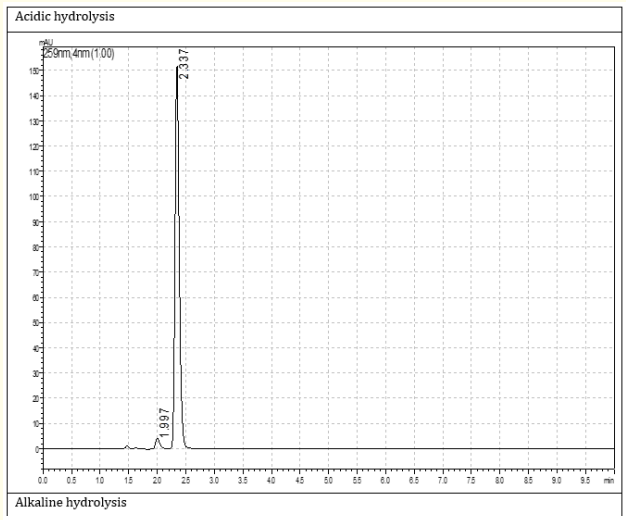
Stress condition	R_t (min)	*Mean peak area	% Recovery*	% Drug degradation	Theoretical Plates (>2000)	Tailing factor (<1.5)	Resolution
Standard drug	2.346	804773	100	-	3809.898	1.384	-
Acidic degradation 0.1N HCl/80°C/30 min	2.313	789535	98.11	1.89	3414.466	1.362	-
Alkaline degradation 0.1N NaOH/80°C/30 min	2.337 1.997	787339	97.83	2.17	3821.374	1.379	
Thermal degradation 80°C/30 min	2.315	787352	97.84	2.16	3603.995	1.386	-
Oxidative degradation H_2O_2/80ºC/30 min	2.326 1.822	782288	97.21	2.79	3512.852	1.351	

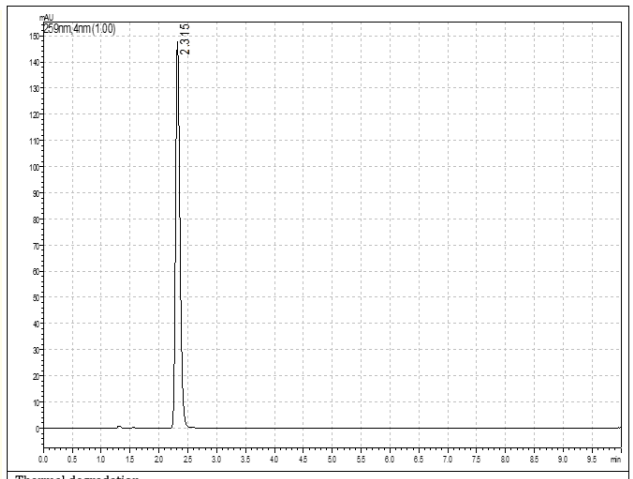
*Mean of three replicates.











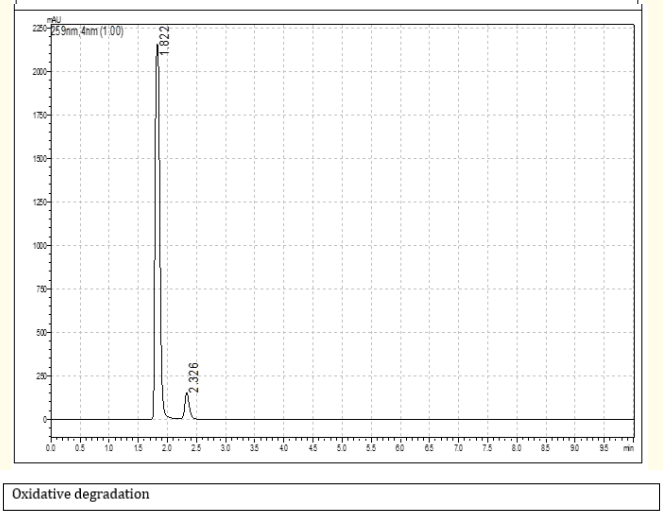


Figure 4: Representative chromatograms of Trifluridine (20 µg/mL) during the forced degradation studies.

Conclusion

A new stability indicating RP-HPLC method has been developed and validated for the estimation of Trifluridine. The method is simple, precise, accurate and robust and the method is very much useful for the routine quantitative analysis of Trifluridine in ophthalmic preparations. The method is selective and specific.

Acknowledgement

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