ORIGINAL ARTICLE

A New Stability Indicating RP-HPLC Method for the Quantification of Trifluridine Using an Ion Pairing Agent

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Abstract

Introduction: Trifluridine is a trifluoro thymidine derivative used to treat viral infections of eyes. A new stability indicating reversed-phase high-performance liquid chromatography (HPLC) method was developed and validated for the determination of trifluridine in pharmaceutical dosage forms (ophthalmic solution). **Materials and Methods:** Shimadzu Model CBM-20A/20 Alite HPLC system with PDA detector and Agilent C18column were used for the present study. Mobile phase mixture consisting of tetra butyl ammonium hydrogen sulfate and methanolin the ratio 48: 52, v/v with a flow rate 1.0 mL/min was chosen for the chromatographic elution of Trifluridine (Detection wavelength 265 nm). **Results and Discussion:** The method was linear over the concentration range 0.1–100 mg/ml with linear regression equation, y = 43421× + 2389.5 (R^2 = 0.9999). The limit of quantitation and limit of detection were found to be 0.0971 mg/ml and 0.0311, respectively. Stress degradation studies were performed and the method was validated as per ICH guidelines. **Conclusion:** The proposed RP-HPLC method is simple, precise, robust, accurate and can be applied for the routine analysis of Trifluridine dosage forms.

Key words: ICH guidelines, reversed-phase high-performance liquid chromatography, stability indicating, stress degradation studies, trifluridine, validation

INTRODUCTION

Trifluridine ($C_{10}H_{11}F_3N_2O_5$) is an anti-viral drug with molecular weight 296.21 g/mol and chemically it is 1-[(2R,4S,5R)-4-hydroxy-5 (hydroxyl methyl) oxolan-2-yl]-5-(tri fluoro-methyl) pyrimidine-2,4-dione. The pharmaceutical formulation (Eye drops) of trifluridine is used for the treatment of keratitis and kerato conjunctivitis of eyes[1,2] caused by herpes simplex virus Type 1 and Type 2.

Mohammad *et al.* have developed a LC-MS/MS technique[3] in human plasma using an internal standard, *β*-thymidine for the estimation of trifluridine. A mobile phase mixture consisting of acetonitrile: methanol: 5 mM ammonium formate (45:40:15) with flow rate 0.8 mL/min with Phenomenex-RP-C18 column was the selected as the chromatographic conditions for the study and Beer-Lambert's law were observed over the concentration 0.005–2.0 µg/ml.

Bandaru and Annapurna have developed a validated stability indicating reversed-phase ultra-fast liquid chromatography (RP-UFLC) method[4] for the estimation of trifluridine using Acetonitrile: Potassium dihydrogen phosphate buffer (10 mM) (adjusted to pH 3.5 with TFA) (70:30) with C18 Shim-pack GWS high-performance liquid chromatography (HPLC) packed column with flow rate 1.0 mL/min (UV detection at 272 nm). The method has shown linearity over the concentration 0.1–120 µg/ml.

Yasaswini *et al.* developed a stability indicating RP-UFLC method[5] 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.

Yasaswini *et al.* have also developed a spectrophotometric[6] methods in borate buffer (pH 9.0), phosphate buffer (pH 7.0, 2.0, 4.0), NaOH, methanol, and water.

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In the present study, the authors have proposed a new stability indicating reversed-phase HPLC (RP-HPLC) method for the estimation of trifluridine using an ion pairing agent and the method was validated as per ICH guidelines.

MATERIALS AND METHODS

Shimadzu Model CBM-20A/20 Alite HPLC system with PDA detector and Agilent C18column was chosen for the present study. Trifluridine API (≥99.0 purity) was obtained from Pfizer Laboratories (India) as gift sample and was used as it is without further purification. HPLC grade methanol (Merck), tetra butyl ammonium hydrogen sulfate (Merck), sodium hydroxide, hydrogen peroxide (30% w/v), and hydrochloric acid were procured from Qualigens (India). HPLC grade water was obtained from Millipore system.

Preparation of 10 mM Tetra butyl ammonium hydrogen sulfate solution (pH 3.35)

Tetra butyl ammonium hydrogen sulfate ($C_{16}H_{37}NO_4S$) is an ion pairing agent. The molecular weight of tetra butyl ammonium hydrogen sulfate is 339.54 g/mole. About 3.3954 g tetra butyl ammonium hydrogen sulfate was accurately weighed and transferred to a 1000 ml volumetric flask and dissolved in HPLC grade water to prepare 10 mM solution and the pH of the resulting solution is 3.35.This buffer solution was filtered through 0.42 micron membrane filter and used for the preparation of mobile phase.

Preparation of stock solution of trifluridine

Standard stock solution of trifluridine was prepared by transferring 25 mg trifluridine in to a 25 mL volumetric flask and diluting with methanol and (1000 µg/mL) and further diluted solutions were prepared using the mobile phase and all these solutions were stored in a refrigerator at 2–8°C.

Instrumentation and chromatographic conditions

Shimadzu Model CBM-20A/20 Alite HPLC system with PDA detector and Agilent C18column was used for the chromatographic study. Mobile phase mixture consisting of tetra butyl ammonium hydrogen sulfate and methanol (48: 52, v/v) with flow rate 1.0 mL/min was the chromatographic conditions chosen for the elution of trifluridine (Detection wavelength 265 nm). The injection volume was 20 µl and the total run time was 10 min.

Method validation[7]

Linearity study

A series of 0.1–100 µg/mL of Trifluridine solutions were prepared from the stock and working standard solutions were prepared on dilution with the mobile phase and each of these solutions was injected *(n* = 3) into the HPLC system and the chromatograms were recorded. The peak area of each of these chromatograms was noted at their retention time and finally the mean peak area was calculated. Calibration curve was drawn by plotting the concentration of trifluridine drug solutions on the X-axis and the corresponding mean peak area on the Y-axis. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the signal to noise ratio (S/N). The LOD is 3.3 times the signal to noise ratio and that of LOQ is 10 times the signal to noise ratio.

Precision study

Precision of the method was evaluated intra-day and inter-day precision studies. Three different concentration solutions (10, 20 and 40 µg/mL) of trifluridine were prepared within the linearity range on the same day (intra-day precision) and on 3 consecutive days (inter-day precision) and the chromatographic study was performed. The mean peak area *(n* = 3) and thereby the % RSD were calculated.

Accuracy study

Accuracy of the method was measured by spiking the drug formulation (20 µg/mL) solution (50, 100, 150%) with a known concentration of standard drug *(n* = 3) where the final concentrations were found to be 30, 40, and 50 µg/mL. The mean peak area was calculated from the chromatograms obtained and finally the % RSD was calculated from the linear regression equation.

Robustness study

The robustness of the method was performed using trifluridine drug solution (10 µg/mL) proved by incorporating a very small changes in the optimized chromatographic conditions such as pH (±0.05; 3.30 and 3.40), mobile phase composition (±2%; 50:50 and 46:54), flow rate (±0.1 mL; 1.1 and 0.9 mL/min), and detection wavelength (±5 nm; 260 and 270 nm).

Assay of Trifluridine

Trifluridine is available as 1% of ophthalmic solution from Pfizer Laboratories (India) and Sandoz Falcon Pharmaceuticals (India). Trifluridine ophthalmic solution of two different

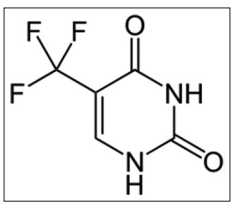

Figure 1: Chemical structure of Trifluridine

brands was procured from the pharmacy store in the local market and the total contents of the formulation were extracted with HPLC grade methanol into a 100 ml volumetric flask and sonicated for 30 min and filtered. The filtered formulation solution was diluted with mobile phase and 20 µL was injected into the HPLC system. The retention time, as well as the peak area of the trifluridine chromatogram eluted, was noted and the percentage purity was calculated from the calibration curve.

Stress degradation studies[8]

Stress degradation studies of trifluridine were performed to determine the stability of trifluridine toward stress conditions such as alkaline hydrolysis, acidic hydrolysis, oxidation, and thermal degradation. The specificity of the method can be known from the stability studies and therefore trifluridine was exposed to the following stress conditions and the stability was studied.

For performing the acidic degradation studies, a specific quantity of trifluridine was transferred into a 10 ml volumetric flask and 1 ml of 0.1N hydrochloric acid solution was added, heated in a thermostat for about 30 min at 80°C, cooled and then neutralized with 1 ml of 0.1N sodium hydroxide and made up to volume with the mobile phase. The contents were mixed and filtered through membrane filter and 20 µl of this solution was injected in to the HPLC system and the peak area of the chromatogram at the retention time was noted and the percentage degradation was calculated from the linear regression equation.

Alkaline degradation was performed by heating trifluridine solution with 1.0 mL 0.1N sodium hydroxide solution in a thermostat for about 30 min at 80°C and the stressed sample was cooled, neutralized with 1.0 mL of 0.1 N HCl solution and made up to volume with the mobile phase. The contents were mixed and filtered through membrane filter and 20 µl of this solution was injected in to the HPLC system and the peak area of the chromatogram at the retention time was noted and the percentage degradation was calculated from the linear regression equation.

Oxidative degradation was performed by heating trifluridine solution with 1.0 mL 30% of hydrogen peroxide solution in a thermostat for about 30 min at 80°C. The stressed sample was cooled and made up to volume with the mobile phase. The contents were mixed and filtered through membrane filter and 20 µl of this solution was injected in to the HPLC system and the peak area of the chromatogram at the retention time was noted and the percentage degradation was calculated from the linear regression equation.

Thermal degradation was performed by heating trifluridine solution with the mobile phase in a thermostat for about 30 min at 80°C and the stressed sample was cooled and made up to volume with the mobile phase. The contents were mixed and filtered through membrane filter and 20 µl of this solution was injected in to the HPLC system and the peak area of the chromatogram at the retention time was noted and the percentage degradation was calculated from the linear regression equation.

RESULTS AND DISCUSSION

A new stability indicating RP-HPLC method has been proposed for the estimation of trifluridine in API and its pharmaceutical formulation, that is, ophthalmic solution using ion pair chromatography and the method was validated as per ICH guidelines.

Shimadzu Model CBM-20A/20 Alite HPLC system with PDA detector and Agilent C18column was employed for the

Table 1: Literature survey of trifluridine

Method	Reagent/Mobile phase (v/v)	Linearity (µg/mL)	Ref
LC-MS/MS (Human plasma) (Internal standard: β-thymidine)	Acetonitrile: Methanol: 5 mM Ammonium formate (45:40:15)	0.005–2.0	3
RP-UFLC (Stability indicating)	Acetonitrile: Potassium dihydrogen phosphate buffer (10 mM) adjusted to pH 3.5 with TFA (70:30)	0.1–120	4
RP-UFLC (Stability indicating)	Acetonitrile: Water (50:50)	1–100	5
Spectrophotometry (D_0& D_1)	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	6
RP-HPLC (Stability indicating)	10 mM Tetra butyl ammonium hydrogen sulfate: Methanol (48:52)	0.1–100	Present method

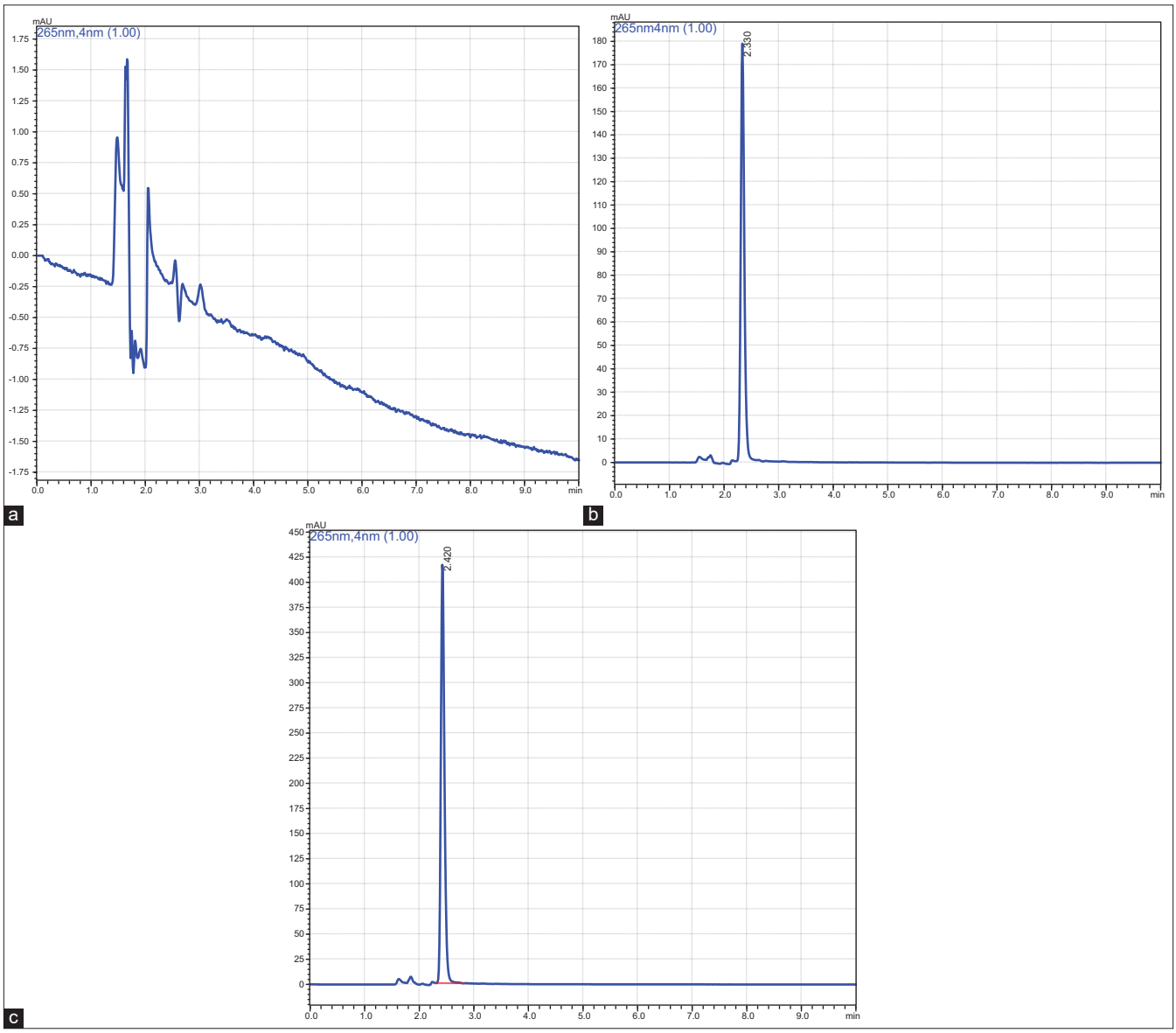

Figure 2: a: Placebo. b: Representative chromatogram of trifluridine standard (API) (20 µg/mL) (Rt 2.330 min) (Theoretical plates: 4795.828) (Tailing factor: 1.351). c: Representative chromatogram of trifluridine ophthalmic solution (50 µg/mL) (Rt 2.420 min) (Theoretical plates:4874.920) (Tailing factor: 1.345). Representative chromatograms of Placebo and Trifluridine

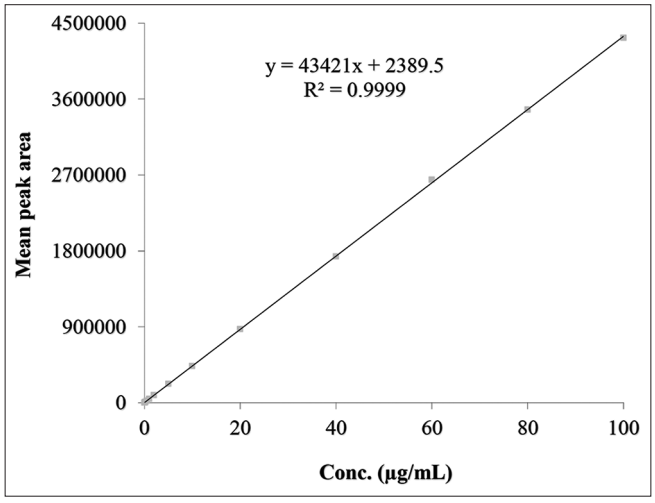

Figure 3: Calibration curve of trifluridine

chromatographic study. Mobile phase mixture consisting of tetra butyl ammonium hydrogen sulfate and methanolin the ratio 48: 52, v/v with a flow rate 1.0 mL/min was chosen for the chromatographic elution of trifluridine (Detection wavelength 265 nm). The method was linear over the concentration range 0.1–100 mg/ml with linear regression equation, y = 43421× + 2389.5 (R^2 = 0.9999). The LOQ and LOD were found to be 0.0971 mg/ml and 0.0311, respectively. The run time was 10 min and the injection volume was 20 µl. Table 1 represents the previously published analytical methods and some of the important parameters were highlighted.

Method optimization

In the literature, few stability-indicating RP-HPLC methods were developed using different columns and

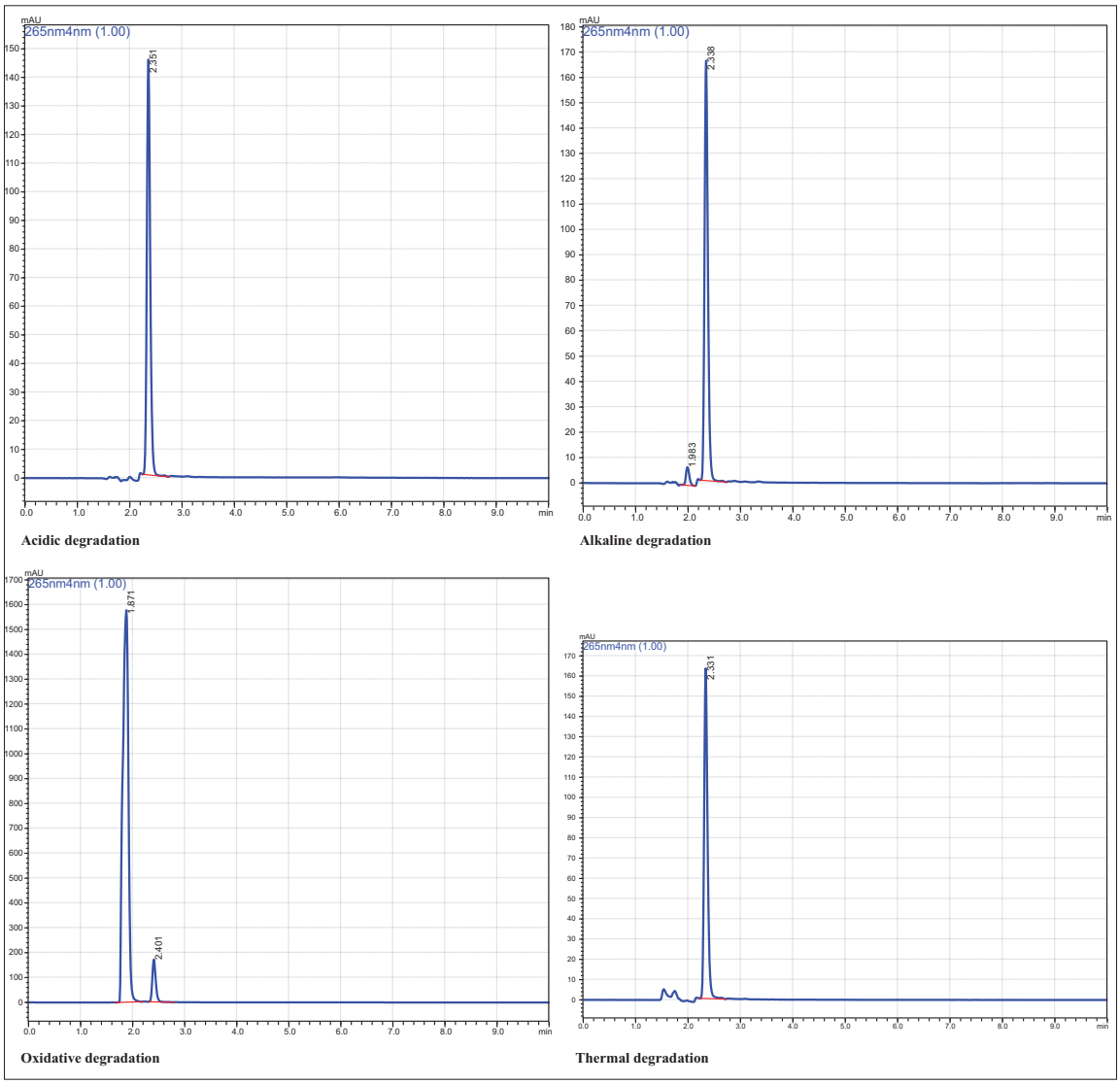

Figure 4: Chromatograms of Trifluridine during stress degradation studies

Table 2: Linearity study of trifluridine

Conc. (µg/mL)	*Mean peak area	% RSD
0	0	0
0.1	4219	0.61
0.2	8739	0.28
0.5	22265	0.54
1	44362	0.48
2	88251	0.34
5	223542	0.35
10	432589	0.64
20	871131	0.72
40	1734543	0.81
60	2642614	0.22
80	3474872	0.85
100	4326475	0.32

*Mean of three replicates

Table 3: Intraday precision study

Conc. (µg/mL)	*Mean peak area	Statistical analysis
		*Mean peak area±SD (% RSD)
10	432589	433498.67±1010.29 (0.2331)
10	434586	
10	433321	
20	871131	871306.67±178.05 (0.0204)
20	871302	
20	871487	
40	1734543	1734303±219.64 (0.0127)
40	1734112	
40	1734254	

*Mean of three replicates

mobile phases and no method has been reported using the ion chromatography technique and therefore the work was initiated with an ion-pairing agent, tetra butyl ammonium

Table 4: Inter-day precision study

Conc. (µg/mL)	*Mean peak area			*Mean peak area±SD (% RSD)
	Day 1	Day 2	Day 3	
10	432589	442157	438214	437653.33±4808.58 (1.0987)
20	871131	869921	872018	871023.33±1052.64 (0.1209)
40	1734543	1740012	1733501	1736018.67±3497.35 (0.2015)

*Mean of three replicates

Table 5: Accuracy study

Spiked drug Conc. (µg/mL)	Drug formulation (µg/mL)	Total drug Conc. (µg/mL)	*Drug recovered (µg/mL)±SD (% RSD)	% Recovery
10 (50%)	20	30	29.81±0.2117 (0.71)	99.37
	20	30		
	20	30		
20 (100%)	20	40	39.39±0.2718 (0.69)	98.48
	20	40		
	20	40		
30 (150%)	20	50	49.83±0.5133 (1.03)	99.66
	20	50		
	20	50		

*Mean of three replicates

Table 6: Robustness study

Parameter	Condition	*Mean peak area±SD (% RSD)
Flow rate (±0.1 ml/min)	1.1	441121±4940.56 (1.12)
	1.0	
	0.9	
Detection wavelength (±5 nm)	270	431598±2632.75 (0.61)
	265	
	260	
Mobile phase composition Tetra butyl ammonium hydrogen sulfate: Acetonitrile (±2%, v/v)	46: 54	439852±3210.92 (0.73)
	48: 52	
	50: 50	
pH (± 0.05 unit)	3.40	437169±4021.96 (0.92)
	3.35	
	3.30	

hydrogen sulfate. 10 mM tetra butyl ammonium hydrogen sulfate solution was used as an aqueous phase and methanol as an organic phase and a 10 µg/mL trifluridine solution was injected initially into the HPLC system using Agilent C_{18}column. Mobile phase consisting of 10 mM tetra butyl ammonium hydrogen sulfate and methanol in 48**:** 52, v/v ratio with flow rate 1.0 ml/min (Detection wavelength 265 nm) was the optimized chromatographic conditions where trifluridine (20 µg/mL) was eluted at 2.330 min and the system suitability parameters (Theoretical plates: 4795.828) (Tailing factor: 1.351) were within the acceptable criteria.

Method validation

Trifluridine obeys Beer-Lambert's law over the concentration range of 0.1–100 µg/mL (% RSD 0.22–0.85) [Table 2]. The representative chromatograms of the placebo and Trifluridine API were shown in Figure 2a and b. The LOQ and LOD were found to be 0.0971 mg/ml and 0.0311, respectively. The linear regression equation was found to be y = 43421× + 2389.5 with correlation coefficient 0.9999 and the calibration curve was shown in Figure 3. The % RSD was found to be 0.0127–0.2331 (Intraday) [Table 3] and 0.1209–1.0987 (Inter-day) [Table 4] in precision studies which is <2.0 indicating that the method is precise. The % recovery in accuracy studies was found to be 98.48–99.76% [Table 5] and % RSD was (0.69–1.03) <2% indicating that the method is accurate. The % RSD in robustness study was also found to be <2% (0.61–1.12) indicating that the method is robust [Table 6].

Assay of trifluridine

The assay of trifluridine ophthalmic solution was performed for two different brands available in the local pharmacy store and the proposed method was applied. The amount of trifluridine present in these pharmaceutical dosage forms was found to be 99.34–99.81 and the excipients of the formulations did not interfere with the trifluridine peak. The chromatogram of the formulation was shown in Figure 2c in which trifluridine

Table 7: Stress degradation studies

Stress conditions	Rt (min)	*Mean peak area	*Drug recovered (%)	*Drug decomposed (%)	Theoretical plates	Tailing factor
Standard drug	2.330	871131	100	-	4795.828	1.351
Acidic degradation	2.351	708880	81.35	18.65	4908.940	1.315
Alkaline degradation	2.338	813168	93.36	6.64	4476.420	1.323
Oxidative degradation	2.401 1.871	774345	88.89	11.11	4836.785	1.341
Thermal degradation	2.331	789847	90.68	9.32	4689.969	1.355

*Mean of three replicates

was eluted at 2.420 min with theoretical plates 4874.920 and tailing factor 1.345 indicating that the system suitability parameters were within the acceptable criteria.

Stress degradation studies

During the chromatographic study, it was observed that trifluridine elutes at 2.3 ± 0.05 min and the system suitability parameters such as theoretical plates were >2000 and tailing factor <1.5 (Acceptable criteria).

During the acidic degradation studies, trifluridine was eluted at 2.351 min with <18.65% degradation with theoretical plates 4908.940 (>2000) and tailing factor 1.315 respectively. The pyrimidine moiety present in the structure of trifluridine may be responsible for this degradation but no degradants were observed.

During the alkaline degradation studies, trifluridine was eluted at 2.338 min and has shown about 6.64% of degradation was observed with theoretical plates 4476.420 (>2000) and tailing factor 1.323 which are within the acceptable criteria.

During the oxidation, trifluridine was eluted at 2.401 min along with a degradant peak at 1.871 min. About 11.11% of degradation was observed with theoretical plates 4836.785 (>2000), resolution 2.982 (>2) and tailing factor 1.2341 indicating that trifluridine is highly resistant towards oxidation.

During the thermal degradation, trifluridine was eluted at 2.331 min and about 9.32% of degradation was observed with theoretical plates 4689.969 and tailing factor 1.355 which are within the acceptable criteria. The method is so specific as no degradants were interfering with trifluridine drug peak. The respective chromatograms so obtained during the stress degradation studies are shown in Figure 4 and the details are shown in Table 7.

CONCLUSIONS

A new stability indicating RP-HPLC method has been developed for the determination of trifluridine and validated as per ICH guidelines. The method is specific and no degradants were interfering with trifluridine peak and there is no interference of excipients of the pharmaceutical formulation. Trifluridine is more sensitive toward acidic degradation. The proposed method is simple precise, accurate, and robust and can be applied for the pharmacokinetic studies.

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