Original Article

Stability Indicating Analytical HPLC Method Development and Validation for the Simultaneous Quantification of Tobramycin and Fluorometholone in Ophthalmic Suspension

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DOI:10.47750/pnr.2023.14.S01.128

Abstract

The combination of tobramycin and fluorometholone was available as eye drop formulation used to treat various bacterial infections of eye like conjunctivitis or pink eye. In literature, there is no stability indicating HPLC method reported for the quantification of tobramycin and fluorometholone. Hence the present study was aimed to establish a simple and sensitive HPLC method for the quantification of tobramycin and fluorometholone in eye drop formulations. The separation of analytes was achieved on spherisorb ODS1 (Waters) C18 (250×4.6 mm; 5 µ id) as stationary phase, acetonitrile: methanol: 0.1% orthophosphoric acid in 45:53:02 (v/v) at pH 4.4 as mobile phase at 0.8 mL/min flow rate. The analytes that were separated in column were detected using UV detector at 219 nm. In this condition, well resolved, retained peaks were identified at 3.4 min for tobramycin and 7.8 min for fluorometholone. The method reports 0.227 µg/mL and 0.076 µg/mL for tobramycin and fluorometholone respectively as LOD that proves that the method have enough sensitivity levels for the detection analytes in samples. The method passes all the validation parameters as per the guidelines proved that the method was valid. The method can shows very less % degradation in various stress studies such as acidic, base, peroxide, thermal and UV light conditions and can effectively separate various stress degradation compounds and confirms the stability indicating nature of the method. The method applicability was assessed by analysing the drug content in eye drops with a % assay of 99.01 % and 98.79 % respectively for tobramycin and fluorometholone. Based on the results, it can be concluded that the method can adequately suitable for the separation and quantification of tobramycin and fluorometholone hence can be applicable for the routine analysis of tobramycin and fluorometholone in eye drops formulations.

Key words: Tobramycin, Fluorometholone, HPLC analysis, Method Development, Forced degradation, Formulation analysis.

INTRODUCTION:

The antibiotic medical drug tobramycin (Figure 1A) belongs to aminoglycoside class that was derived from *Streptoalloteichus tenebrarius* bacterium that was prescribed to treat different types of infections caused by bacteria especially gram negative bacteria [1]. It shows potentially effective against *Pseudomonas* species. It was used for the treatment of bacterial infections of lower respiratory tract, skin structure, skin, bone and urinary tract [2,3].

It was also used in treating eye superficial infections like bacterial conjunctivitis. Like other aminoglycosides drugs, ototoxicity (toxic to ear) and equilibrioception (perception of balance) are the major side effect possible during the usage of tobramycin. The other side effects includes hypersensitivity reactions, nephrotoxicity and neuromuscular toxicity are possible side effects of tobramycin [4].

Fluorometholone (Figure 1B) belongs to corticosteroid or the steroid medicine that reduce the inflammation to eyes. It was suggested to relief inflammation at palpebral and bulbar conjunctiva, cornea and anterior segment of eye globe. It was also used for treating seasonal allergic conjunctivitis and other eye allergies [5,6].

During the usage of fluorometholone, there is a risk of Hypothalamic-pituitary-adrenal (HPA) suppression and rise of intraoccular pressure [7]. Eye pain, burning, stinging, redness, swelling, increased tears, blurry vision and inflammation are the side effects associated with the use of fluorometholone.

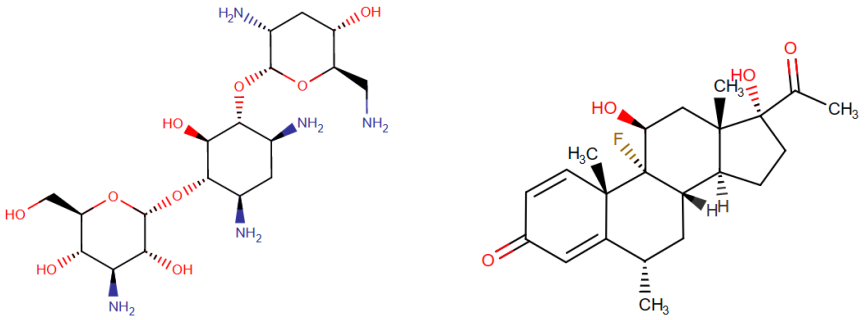


Figure 1: Molecular structure of Tobramycin (A) and Fluorometholone (B)

The combination of tobramycin and fluorometholone was available as ophthalmic formulation and was prescribed for the treatment of common eye infections caused by bacteria that was associated with inflammation of eye white part and eyelid. This ophthalmic eye dose combination was available with strength of 0.3% (w/v) of tobramycin and 0.1% (w/v) of fluorometholone. Blurred vision, irritation, itching, burning and redness of eye are side effects possible during the usage of the ophthalmic combination of tobramycin and fluorometholone. Figure 1 presents the molecular structure of tobramycin and fluorometholone.

The extensive review of the literature was conducted for the evaluation of available analytical methods for estimating tobramycin and fluorometholone in pharmaceutical formulations using different analytical techniques. In the literature review, it was observed that few analytical methods reported for analyzing tobramycin [8,9,10] in single or in combination with loteprednol [11], dexamethasone [12] and vancomycin [13] in formulations. Methods also reported for the estimation of fluorometholone in single [14,15,16], in combination with tetrahydrozoline [17], sodium cromoglycate [18], tetrahydrozoline [19] and ketorolac tromethamine [20] in various samples. Based on the literature review, it was confirmed that there is no HPLC method published for the quantification of tobramycin and fluorometholone. Hence the present work intended to develop a simple stability indicating HPLC method for the simultaneous quantification of tobramycin and fluorometholone in ophthalmic formulations.

MATERIALS AND METHODS:

Chemicals and reagents:

The API of tobramycin and fluorometholone with purity of 98.93 % and 99.31 % were obtained from Mankind Pharma Private Limited, Hyderabad, Telangana. The eye drop formulation of tobramycin and fluorometholone at dosage of 0.3% (w/v) and 0.1% (w/v) respectively with brand name Toba F® was purchased from local pharmacy. The solvents used in the study such as methanol, acetonitrile were of HPLC grade and Milli-Q® was procured from Merck chemicals, Mumbai. The chemicals such as hydrogen peroxide, sodium hydroxide, hydrochloric acid and buffer chemicals were also procured from Merck chemicals, Mumbai.

Instrumental conditions:

The study was conducted on Agilent (USA) 1100 HPLC instrument that comprises of G1311 Aquaternary pump for delivery of solvents, 0.1 – 1500 µL volume injectable auto-sampler with thermostat and UV detector (G 1314 A). Various configurations of stationary phases were used for the method development studies and the column eluents were integrated using Agilent chem-station software.

Preparation of standard solutions:

An exactly weighed 25 mg of tobramycin and fluorometholone were dissolved in 25 mL clean and dry volumetric flask. Then 25 mL of methanol was added separately in each flask and sonicate the flasks for 2 min to dissolve the analytes completely in the solvent. Then the content was filtered through 0.2 µ membrane filter in a separate clean and dry flask separately and the final volume was made up to the mark with the same solvent. The tobramycin and fluorometholone standard solution at a concentration of 1000 µg/mL was obtained separately. The combined standard solutions were prepared by accurately mixing equal volumes of individual known standard stock solutions in a separate flask and were used for method development and validation study [21].

Preparation of test solution:

The Toba F® (0.3 % w/v of tobramycin and 0.1 % w/v of fluorometholone) eye drop formulation was used for the preparation of sample solution. An accurately pipetted one mL of the formulation pipetted in a 10 mL volumetric flask containing 5 mL of methanol. The solution was sonicated for 2 min using ultrasonic bath sonicator and filtered through 0.2 micron membrane filter in to a clean and dry 10 mL volumetric flask. The final volume was made up to the mark using same diluent and the formulation stock solution at 200 µg/mL, 300 µg/mL and 100 µg/mL of tobramycin and fluorometholone respectively. The formulation stock solution was further diluted to required concentration using the same diluent and the selected concentration solution was used for the quantification of tobramycin and fluorometholone in formulation sample [22].

Method development:

The systematic method development steps as prescribed in ICH guidelines [23] were adopted for the development of analytical method for the simultaneous quantification of tobramycin and fluorometholone. Prior to the development of method, the UV detector wavelength was initially identified by using UV-visible spectrophotometer. The iso-absorption wavelength of tobramycin and fluorometholone was considered as suitable wavelength for the detection of both analytes simultaneously using UV detector during HPLC analysis. The flow rate of the mobile phase was initially set as 1.0 mL/min and later optimized in the flow range of 0.5 mL/min to 1.5 mL/min. As both analytes were polar compounds, the non-polar stationary phases were studied for the separation of analytes. The highly non-polar C18 columns with various manufactures were studied for effective separation of analytes. The mobile phase optimization was studied based on the elution of peaks in each studied condition. Various compositions of mobile phase solvents such as methanol, acetonitrile with or without buffer were studied.

In all the method development conditions studied, the standard solution containing 60 µg/mL of tobramycin and 20 µg/mL of fluorometholone was injected and the chromatographic response was recorded. The peak area response, peak intensity, peak shape and the system suitability was summarized in all the studied conditions. The method conditions that produce best system suitability with high peak intensity and significantly no noise was considered as suitable conditions for the separation and analysis of tobramycin and fluorometholone. These developed method conditions were further studied for method validation study.

Method Validation:

The standard solution containing tobramycin at 60 µg/mL and fluorometholone at 20 µg/mL was analysed in the optimized method and the chromatographic response of the resultant chromatograms was summarized for evaluating system suitability. The blank (diluent only), placebo solution prepared with commonly used formulation excipients was analysed in the developed method for evaluating method specificity. A series of dilution of tobramycin and fluorometholone was prepared in various concentration levels. The prepared dilutions was analysed in the developed method and the peak area response of standard and both the impurities were tabulated separately. The calibration curve was constructed for tobramycin and fluorometholone separately by taking the peak area response of analyte in y-axis and its concentration on x-axis. The correlation coefficient and the regression equation of standard tobramycin and fluorometholone were derived from its corresponding calibration graphs.

The method accuracy was evaluated by performing the spiked recovery study and was performed at 50%, 100% and 150% spiked levels. The spiked level solution of tobramycin and fluorometholone was spiked to 100 % formulation solution and the recovery solution was analysed in the optimized method. The peak area response of the recovery solution was compared with the calibration curve results in the same level and the % recovery of each analysis results and in each spiked level the % relative standard deviation (% RSD) was calculated. The % recovery of 98-102 and % RSD of < 2 was considered as acceptable.

The reproducibility of the method was evaluated in terms of precision and was carried as intraday and interday precision. In this, the standard containing 60 µg/mL of tobramycin and 20 µg/mL of fluorometholone was assessed six times in one day for intraday precision and 6 times in three consecutive days for interday precision. The peak area response of standard and both impurities was tabulated and the %RSD of the peak area response was calculated. The %RSD of less than 2 in both the precision studies for all the analytes was considered as the method was precise and repeatable.

The efficiency of the developed method that remains unaffected when there is a small change in the established method conditions as well as the change in analyte was assessed in ruggedness and robustness study. In ruggedness, the solution at precision level was prepared and analysed by three different analysts and the peak area values were tabulated and % RSD of < 2 was acceptable. In robustness study, both positive and negative minor variations in the established method conditions made intentionally and the standard solution at precision level was analysed in each changed condition. The % change in peak area of each analyte in each changed condition was determined and a value of < 2 was acceptable.

The smallest analyte concentration that can detect and quantify in the established method was considered as limit of detection and quantification respectively. This information of the method confirms its sensitivity. The signal (s) to noise (n) ratio method was adopted for the evaluation of sensitivity.

The stability indicating nature of the method was assessed by performing stress degradation studies and the stress studies such as acidic, base, peroxide, thermal and UV light degradation studies was performed to the standard drug. An accurately weighed 50 mg of standard tobramycin and fluorometholone was mixed separately with 50 mL of hydrochloric acid (0.1 N), sodium hydroxide (0.0 N) and hydrogen peroxide (3%) in acid, base and peroxide degradation studies respectively. The solutions were incubated for 24 h in dark, neutralized and then bring it to standard concentration prior to the analysis. The standard tobramycin and fluorometholone was exposed to 60 ^{0}C for 24 h in an air oven and UV light at 254 nm for 24 h in thermal and UV light degradation studies respectively. Both these standard drugs after stress exposer were diluted to standard concentration prior to the analysis. All the stress exposed dilute solutions were evaluated in the established method and the chromatograms observed in each analysis were observed for confirming the acceptability of the method. The resultant chromatograms provides the number of stress degradation compounds generated as a results of stress exposer and the method applicability for the separation of stress degradation compounds was assessed. The peak area in each stress study was used for calculating the % degradation of tobramycin and fluorometholone by comparing with un-stressed peak area response in the developed method.

The developed method was applied for the separation, detection and quantification of tobramycin and fluorometholone in formulation. The formulation sample solution prepared from Toba F® eye drops was assessed in the developed method. The peak area response was used to calculate the % content in the sample by comparing with corresponding standard calibration curve results.

RESULTS AND DISCUSSIONS:

The individual standard solution of tobramycin and fluorometholone was scanned in the range of 400-200 nm and the overlay UV absorption spectra confirm the iso-absorption wavelength that suitable for the detection of both analytes. Based on the study results, at a wavelength 219 nm was proved to be suitable for the detection of tobramycin and fluorometholone. Hence method development was performed by fixing detector wavelength as 219 nm.

The stationary phase, mobile phase composition with wide pH range was studied during the method development study. The method development trails performed and results achieved in the trails performed was summarized in table 1. The chromatograms identified during the method development were presented in figure 2

S No	Method condition	Results	Figure
1	MP: methanol: water in 80:20; SP: Zodiac 250 mm C18 column; pH: 5.8; WL: 219 nm	No clear separation of analytes was observed. Peak corresponds to fluorometholone was not identified and baseline disturbances was observed	2A
2	MP: acetonitrile: water in 75:25; SP: Kromasil 250 mm C18 column; pH: 5.2; WL: 219 nm	Peak corresponds to fluorometholone was identified and the peak area response and peak intensities of both analytes was observed to be very less. Baseline was disturbed throughout the run time.	2B
3	MP: acetonitrile: methanol in 35:65; SP: Cosmosil 250 mm C18 column; pH: 5.1; WL: 219 nm	Both analyte peaks were identified and fluorometholone peak intensity and symmentry was higher than tobramycin. The baseline distrubances was decreased compared with previous trail	2C
4	MP: acetonitrile: methanol: 0.1% orthophosphoric acid in 45:53:02; SP: Spherisorb 250 mm C18 column; pH: 4.8; WL: 219 nm	Both analyte peaks were identified but the resolution betwwen the identified peaks were very less. Clear baseline was observed.	2D

MP: mobile phase; WL: wavelength; SP: column

Table 1: results observed during the HPLC method development

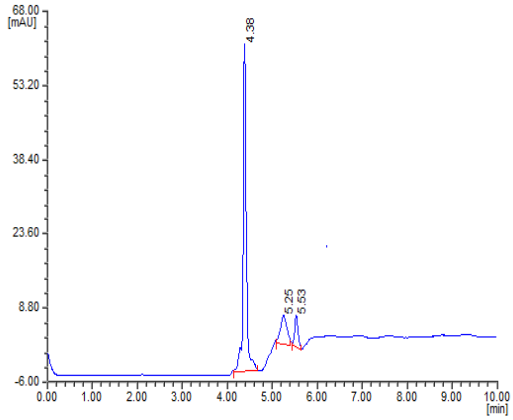


Fig. 2A

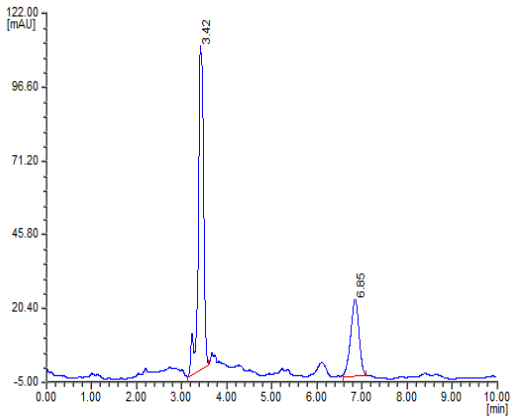


Fig. 2B

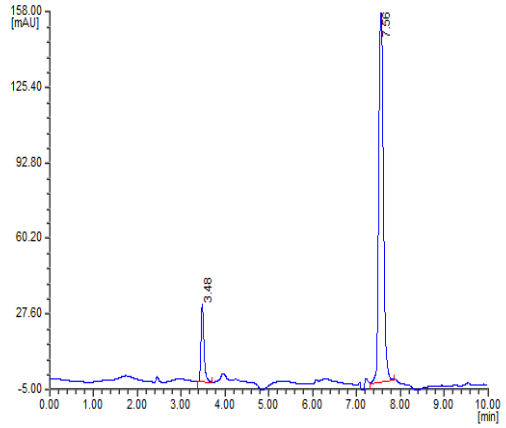


Fig. 2C

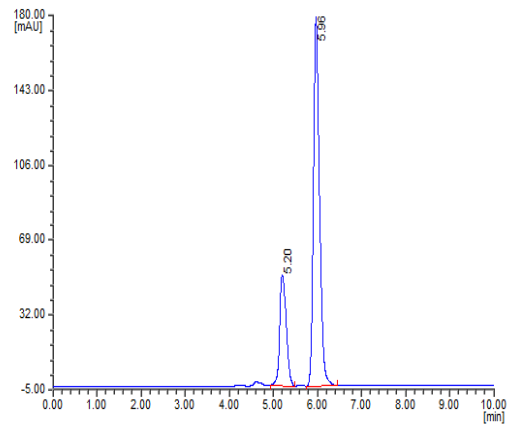


Fig. 2D

Figure 2: Method development Chromatograms in the study

The method development was concluded after achieving the suitable conditions for the separation of tobramycin and fluorometholone with acceptable symmetry and system suitability. Based on the results achieved, best separation was achieved using spherisorb ODS1 (Waters) C18 (250×4.6 mm; 5 µ id) as stationary phase, acetonitrile: methanol: 0.1% orthophosphoric acid in 45:53:02 (v/v) at pH 4.4 as mobile phase at 0.8 mL/min flow rate. The analytes that were separated in column were detected using UV detector at 219 nm. In these optimized chromatographic conditions, clear separation of tobramycin and fluorometholone was achieved with no additional detection of impurities or other co-eluting compounds. The analytes were identified at a retention time of at a retention time of 3.4 min for tobramycin and 7.8 min for fluorometholone whereas the chromatogram of blank doesn't show any chromatographic detections throughout the run time. This confirms that the established method was specific for the detection of tobramycin and fluorometholone. The chromatogram of blank and standard observed in the developed method condition was represented in figure 3A and 2B respectively.

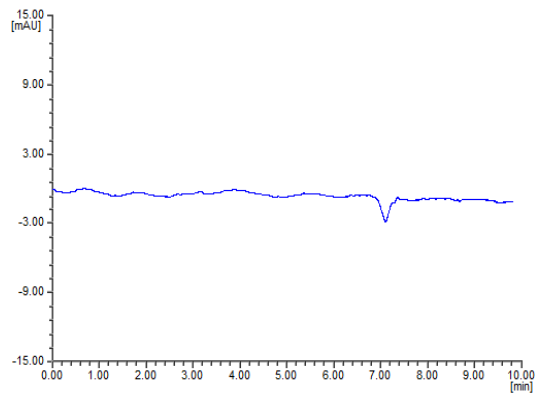


Fig 3A: Blank chromatogram

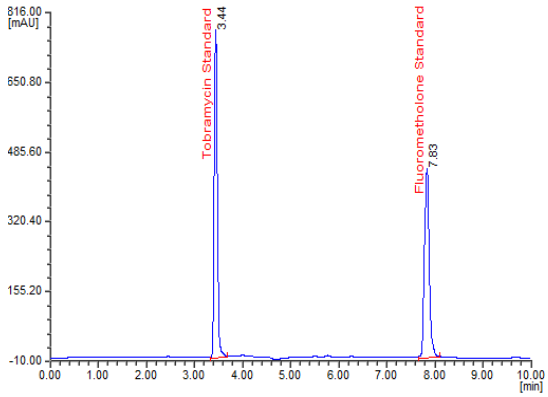


Fig 3B: Standard chromatogram

Figure 3: System suitability chromatograms observed in optimized conditions

The standard calibration curve solutions of tobramycin and fluorometholone was prepared and analysed in the optimized method. The high correlated calibration curve was attained in the analyte range of 15 – 90 µg/mL for tobramycin and 5 – 30 µg/mL for fluorometholone. The regression equation derived as y = 8688.2x - 9574.2 (R^2 = 0.9998) tobramycin and y = 13931x – 12578 (R^2 = 0.9998) for fluorometholone. The peak area results identified in the linearity study were represented in Table 2.

S. No	Tobramycin		Fluorometholone	
	Concentration in µg/mL	Peak Area	Concentration in µg/mL	Peak Area
1	15	121547.9	5	59351.8
2	30	249003.5	10	125325.1
3	45	381141.8	15	194690.3
4	60	511014.9	20	264813.5
5	75	648470.3	25	337481.9
6	90	768151.5	30	405615.1

Table 2: Linearity results

The standard solution containing 60 µg/mL of tobramycin and 20 µg/mL of fluorometholone was assessed in the optimised method for evaluating system suitability. The system suitability parameters of the chromatographic results were summarized and the method system suitability was assessed. As summarized in table 3, the developed method passes the system suitability confirms the suitability of the developed method.

S No	Parameter	Results achieved for	
		Tobramycin	Fluorometholone
1	Analyte strength	60 µg/mL	20 µg/mL
2	Retention Time	3.4 min	7.8 min
3	Theo plate	6914	9749
4	Tail Factor	1.05	0.94
5	Resolution	--	12.61

Table 3: System suitability results

The standard solution at 60 µg/mL of tobramycin and 20 µg/mL of fluorometholone was evaluated in precision and ruggedness study. The peak area response of each analyte was summarized in each study and the % RSD was calculated as 0.24 and 0.20 in intraday precision, 0.81 and 1.06 in interday precision and 0.43 and 0.51 in ruggedness for tobramycin and fluorometholone respectively. The % RSD was achieved under the acceptable levels for all the analytes in each study proved that the method was precise and rugged.

The influence of the variations in the developed method conditions on the chromatographic response was assessed in robustness study. In robustness study, the composition of mobile phase was altered as acetonitrile: methanol: 0.1% orthophosphoric acid in 40:58:02 (v/v) in MP change 1 and 50:48:02 (v/v) in MP change 2. The pH of buffer was altered as 4.3 (pH 1) and 4.5 (pH 2) as well as the detector wavelength of changed as 224 nm (WL 1) and 214 nm (WL 2). The % change in the peak area response of individual analyte was calculated and results reported under the acceptable levels. The system suitability of the individual analyte in each changed conditions was summarized (Table 4) and results reported under the acceptable levels confirms that the method was rugged.

S No	Compound	Change	Peak Area	% Change	Plate Count	Tail factor	Resolution
1	Tobramycin	MP 1	516098.4	0.99	6991	1.04	--
2		MP 2	514494.2	0.68	6945	1.05	--
3		pH 1	506614.6	0.86	6955	1.03	--
4		pH 2	505618.1	1.06	6973	1.05	--
5		WL 1	508435.4	0.50	6929	1.04	--
6		WL 2	504763.6	1.22	6974	1.03	--
7	Fluorometholone	MP 1	262977.1	0.69	9812	0.94	12.66
8		MP 2	264021.9	0.30	9784	0.94	12.65
9		pH 1	263924.3	0.34	9719	0.93	12.61
10		pH 2	264307.5	0.19	9802	0.94	12.63
11		WL 1	263565.7	0.47	9784	0.93	12.62
12		WL 2	263782.3	0.39	9843	0.95	12.63

Table 4: Robustness results

The spiked recovery was performed to evaluate the precision of the developed method and recovery study was performed by spiked recovery that was conducted in 50%, 100% and 150%. The standard solution in the linearity study i.e 10 µg/mL of tobramycin and 25 µg/mL of fluorometholone was considered as target concentration in spiked recovery study. The solutions were evaluated in the optimized method and the peak area response was compared with the standard calibration results in the same level. The % recovery for each injection and the % RSD in each spiked level was calculated. The % recovery was observed to be with in the acceptable levels of 98-102 % and the % RSD was observed to be less than 2 in each spiked level (Table 5) confirms the method accuracy.

S. No.	Compound	Recovery Level	Concentration prepared in µg/mL	Amount found* Mean ± SD	% Recovered* Mean ± SD	% RSD of Recovery
1	Tobramycin	50 %	15	14.86±0.080	99.08±0.533	0.54
2		100 %	20	19.74±0.078	98.69±0.391	0.40
3		150 %	25	25.07±0.152	100.27±0.607	0.61
4	Fluorometholone	50 %	12.5	37.07±0.169	98.86±0.450	0.45
5		100 %	25	49.74±0.128	99.47±0.255	0.26
6		150 %	37.5	62.80±0.257	100.48±0.411	0.41

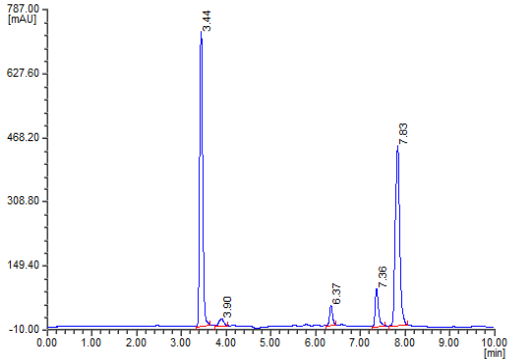
* n=3

Table 4: Recovery results

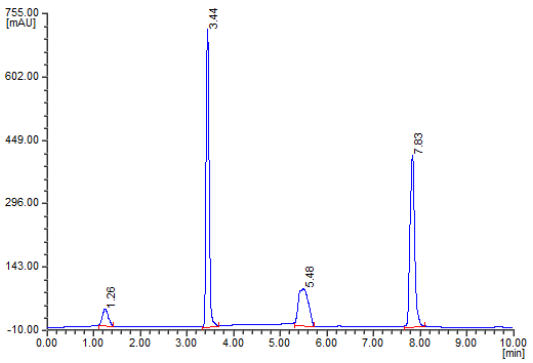
The sensitivity of the established method was assessed by evaluating the limit of detection (LOD) and limit of quantification (LOQ) that was performed by adopting s/n approach. The LOD was conformed as 0.227 µg/mL and 0.076 µg/mL for tobramycin and fluorometholone. Based on LOD, the LOQ was calculated as 0.75 µg/mL and 0.25 for tobramycin and fluorometholone. This confirms that the method can effectively detect the impurities up to very low concentrations for all the analytes.

The method was evaluated for its applicability for the separation and analysis of various compounds generated due to stress degradation of tobramycin and fluorometholone. The standard drug was exposed to various stress conditions and then the stressed sample was evaluated in the developed method. The resultant chromatograms and its results were analysed for the evaluation of its applicability for the separation of stress degradants. Peaks correspond to tobramycin and fluorometholone was identified at its standard retention time along with three additional degradation compounds.

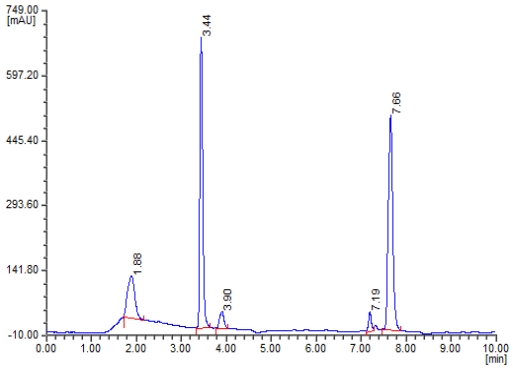
Well resolved and retained three additional degradation products were identified at a retention time of 3.9 min, 6.3 min and 7.3 min. The % degradation was observed to be 5.24 % and 6.17 % respectively for tobramycin and fluorometholone. In base degradation study, a very less % degradation of 3.11 % and 2.91 % was observed respectively for tobramycin and fluorometholone with two additional products identified at 1.2 min and 5.4 min. Three additional degradation products were well retained and identified along with tobramycin and fluorometholone in peroxide, thermal and UV light degradation study separately. The % assay of both analytes was observed to be high and both the analytes were well resolved and retained in all these stress studies. There is no detention of unwanted compounds or the impurities identified detected in the chromatograms observed in the stress studies confirms that the method can effectively separate the degradation compounds during the stress degradation of both the analysis proves that the method was stability indicating. Figure 4 represents the chromatograms obtained during the stress studies of tobramycin and fluorometholone in the developed method.



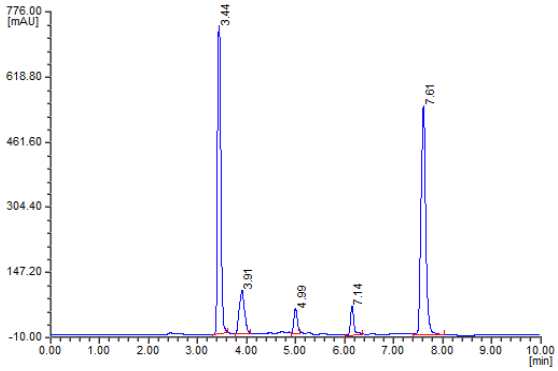
Acid degradation



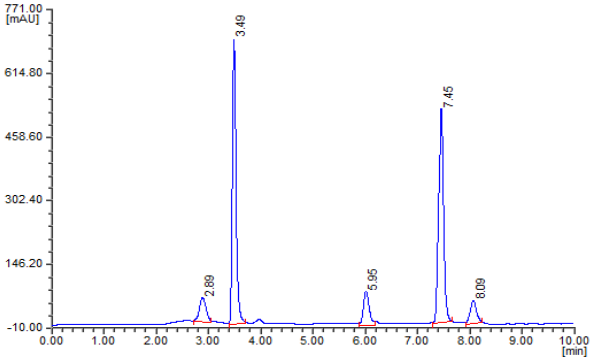
Base degradation



Peroxide degradation



Thermal degradation



UV Light degradation

Figure 5: Forced degradation chromatograms

The analytical method optimized in the study was applied for its applicability for the estimation of tobramycin and fluorometholone in eye drops. The formulation solution prepared using Toba F® was used for the formulation assay study. The resultant chromatogram show clear identification and resolution of tobramycin and fluorometholone. The % assay was observed to be 99.01 % and 98.79 % respectively for tobramycin and fluorometholone. The formulation chromatogram (Figure 6) clearly shows no detection of impurities or additional unwanted compounds as well as the formulation excipients. This confirms that the method was significantly used for the evaluation of tobramycin and fluorometholone in eye drop formulations.

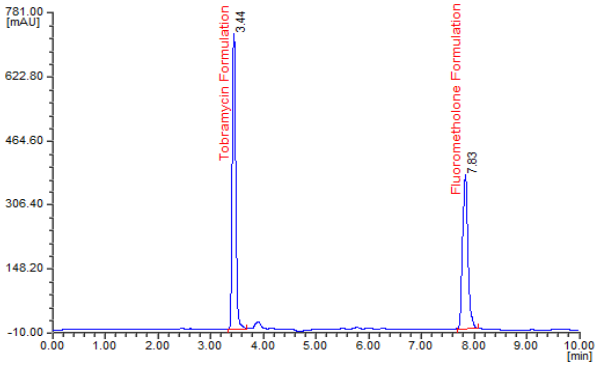


Figure 6: Formulation chromatogram

CONCLUSION:

A simple and novel stability indicating analytical RP-HPLC method was optimized for separation and quantification of tobramycin and fluorometholone in eye drop formulations. The method reports very sensitive detection limit of 0.227 µg/mL and 0.076 µg/mL for tobramycin and fluorometholone confirms that the method can detect the analytes at very low levels. The other validation parameters such as specificity, system suitability, accuracy/recovery, repeatability and reproducibility results were under the acceptable level. The method can efficiently resolve, detect and quantify unknown stress degradation products along with the analytes. Based on the obtained validation results and method application studies, it can be concluded that the method can effectively utilized for analysing tobramycin and fluorometholone in stress samples, bulk drug as well as eye drop formulations.

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