STABILITY INDICATING ANALYTICAL HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF FLUOROMETHOLONE, NEOMYCIN AND BENZALKONIUM CHLORIDE IN OPHTHALMIC SUSPENSIONS

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Abstract:
The ophthalmic suspension of fluorometholone, neomycin and benzalkonium chloride was prescribed for the treatment of inflammatory conditions of the eye, infectious conjunctivitis, as well as bacterial eye infections. As the literature doesn't show any analytical method for the estimation of these drugs in ophthalmic formulations and hence this study intended to develop a simple and and selective HPLC method for the separation and simultaneous quantification of fluorometholone, neomycin and benzalkonium chloride in ophthalmic formulations. The separation of fluorometholone, neomycin and benzalkonium chloride was achieved on Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm) column as stationary phase, acetonitrile and ammonium formate in 0.1% aqueous formic acid in the ratio of 65:35 (V/V) at pH 5.2 as mobile phase. The mobile phase was pumped in isocratic mode at a flow rate of 0.6 mL/min. The separated compounds were detected using UV detector at a wavelength of 237 nm. In the optimised conditions, the retention time was observed at 3.41 min, 4.63 min and 5.89 min respectively for fluorometholone, neomycin and benzalkonium chloride. The method was found to be very sensitive with detection limit of 0.313, 1.094 and 0.013 µg/mL for fluorometholone, neomycin and benzalkonium chloride respectively. Good linear correlation was observed wit in the concentration range of 2.5–15 µg/mL, 8.75-52.5 µg/mL and 0.10–0.60 µg/mL for fluorometholone, neomycin and benzalkonium chloride respectively. The results of stress degradation studied carried in acidic, base, peroxide, thermal and UV light degradation conditions confirms that the method can effectively separates stress degradation compounds and the % degradation was found to be less than 10% in all the stress degradation conditions studied. Hence the method can effectively utilise for the routine analysis of fluorometholone, neomycin and benzalkonium chloride in ophthalmic formulation as well as stability studies.

Keywords: Fluorometholone, Neomycin and Benzalkonium chloride, HPLC analysis, Method Development, Stress studies, Formulation assay.

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Introduction:

Fluorometholone (Figure 1A) is an ophthalmic corticosteroid used for the relief of inflammation located in both the palpebral and bulbar conjunctiva, the cornea, and the anterior segment of the globe of the eye [1]. It reduces the symptoms such as swelling, redness, and itching in eyes [2]. It has also been used topically in the treatment of various skin disorders [3]. The side effects possible during the usage of fluorometholone are pain behind your eyes, sudden vision changes, slow healing after your eye surgery, eye pain, tunnel vision, seeing halos around lights, and signs of a new eye infection [4].

Neomycin (Figure 1B) is an aminoglycoside antibiotic agent used orally and topically to treat a wide variety of infections in the body [5]. Neomycin prevents bacterial infection in the intestines and also used to reduce the symptoms of hepatic coma [6]. Neomycin has also been used to treat small intestinal bacterial overgrowth. Neomycin is not administered via injection, as it is extremely nephrotoxic (damaging to kidney function) even when compared to other aminoglycosides [7]. The common side effects of neomycin include irritation or soreness of the mouth or rectal area, nausea and vomiting. Any loss of hearing, clumsiness, diarrhea, difficulty in breathing, dizziness, drowsiness, greatly decreased frequency of urination or amount of urine, increased amount of gas, increased thirst, light-colored, frothy, fatty-appearing stools, ringing or buzzing or a feeling of fullness in the ears are side effects possible while using neomycin [8].

Benzalkonium Chloride (Figure 1C) is a quaternary ammonium compound uses as Phase transfer agent, cationic surfactant and bioactive agent [9]. It was used as preservative in the preparation of various pharmaceutical products eye, ear and nasal sprays or drops. It was also used in the preparation of wet wipes, hand sanitizers, soaps, shampoos, deodorants, cosmetics, throat lozenges, mouthwashes, spermicidal creams, skin antiseptics and wound wash sprays [10,11]. It has global significance and applicability as preservative in ophthalmic preparations but its shows significant toxicity and irritant properties. Hence companies are focusing to prepare preservative free preparations or replace benzalkonium chloride with less harm preservative [12].

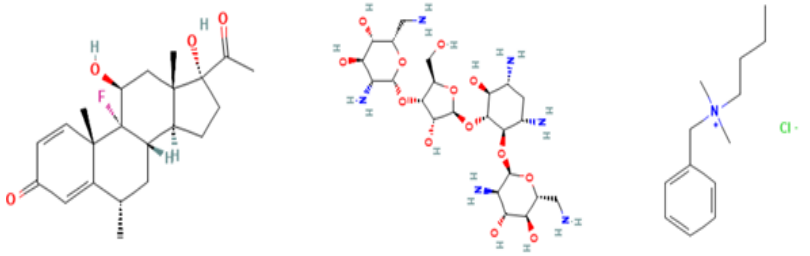


Figure 1: Molecular structure of analytes in the study

The ophthalmic suspension of fluorometholone and neomycin sulphate containing benzalkonium chloride as preservative was used to treat inflammatory conditions of the eye, infectious conjunctivitis, as well as bacterial eye infections. It was also prescribed to treat the eye after the removal of foreign substances. It is used for the treatment of conjunctivitis (sticky eyes) due to organisms sensitive to neomycin. It can be used for the treatment of infections at the front of the eye by bacteria sensitive to the antibiotic neomycin. It can also be used following removal of something from eye, as well as before and after surgery, where there is a possibility of infection with susceptible organisms [13].

The available analytical methods prove that there is no analytical method reported for the simultaneous estimation of fluorometholone, neomycin and benzalkonium chloride in eye drop formulations. One HPLC method reported for the estimation of fluorometholone and neomycin in eye drop formulations [14]. Few analytical methods reported for estimation of fluorometholone in single [15] or in combination with other drugs such as cromoglycate, tetrahydrozoline and ketorolac in formulations [16-20]. Few analytical methods were available for the estimation of neomycin in formulations [21-24]. Based on the literature review, it was confirmed that there is no HPLC method published for the quantification of fluorometholone, neomycin and benzalkonium chloride. Hence the present work intended to develop a simple stability indicating HPLC method for the simultaneous quantification

of fluorometholone, neomycin and benzalkonium chloride in ophthalmic formulations.

Materials and Methods:

Chemicals and reagents:

The pure compounds of fluorometholone (98.57%), neomycin (98.86) and benzalkonium chloride (98.62 %) were obtained from Allergan India Private Limited, Bengaluru, Karnataka and its ophthalmic formulation solution with brand Flurin-N® (0.1% of fluorometholone, 0.35% of neomycin sulphate and 0.004% of benzalkonium chloride) were purchased from local market. The HPLC grade methanol, acetonitrile and ultra-pure (Milli-Q®) were purchased from Merck chemicals, Mumbai. The analytical reagent grade chemicals like hydrogen peroxide, sodium hydroxide, hydrochloric acid and buffer chemicals were also purchased 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 solutions:

***Standard solutions*:**

The stock solution of fluorometholone, neomycin and benzalkonium chloride were prepared separately by mixing 50 mg of compound in 50 mL methanol solvent. Then selected concentration of fluorometholone, neomycin and benzalkonium chloride prepared separately and equal volumes of known concentrations were mixed while performing the experiment.

***Formulation solution*:**

The ophthalmic suspension of Flurin-N® brand containing 0.1% of fluorometholone, 0.35% of neomycin sulphate and 0.004% of benzalkonium chloride) was used for the preparation of formulation solution. An accurately pipetted 1 mL of formulation suspension was mixed to 5 mL methanol in a 10 mL volumetric flask. The content was mixed using sonicator and filtered through 0.45 µ membrane filter. Then it was diluted to 100 % concentration in the linearity level while doing the formulation analysis.

Method development:

Prior to the development of the method, the UV iso-absorption wavelength of fluorometholone, neomycin and benzalkonium chloride was determined using UV spectrophotometer. The composition, flow rate, pH of the mobile phase, stationary phase was optimised by change in different conditions and analysing the standard solution containing fluorometholone, neomycin and benzalkonium chloride. The peak area response, system suitability conditions of the resultant chromatograms were observed for selecting the suitable conditions for validation [25-27].

Method Validation:

The placebo, blank and standard solution containing 100 % concentration of fluorometholone, neomycin and benzalkonium chloride was analysed in the developed method for determining the system suitability and specificity of the method. The standard solution at 25% to 150 % concentration of fluorometholone, neomycin and benzalkonium chloride was analysed for determining the linearity range of the analysis. Calibration curve was constructed by considering the peak area of each analyte against concentration prepared. In the calibration range, the spiked recovery was performed at 50 %, 100 % and 150 % spiked levels. The % recovery for fluorometholone, neomycin and benzalkonium chloride was calculated and a % recovery 98-102 was considered to be acceptable. The known standard solution of fluorometholone, neomycin and benzalkonium chloride at 100 % concentration level was analysed six times in the same day for intraday precision, six times in three different days for interday precision and six times by three different analysts for ruggedness study. The % relative standard deviation of the peak area response of fluorometholone, neomycin and benzalkonium chloride was calculated for evolution and % RSD of less than 2 was considered as the precise and rugged nature of the method. The robustness of the method was confirmed by determining the change in the separation and analysis of fluorometholone, neomycin and benzalkonium chloride when small changes made in the developed method conditions. The % change in the peak area response and the system suitability conditions of the resultant chromatograms was evaluated. The standard drug was exposed to acid (50 mg in 50 ml of 0.1 N HCl), base (50 mg in 50 ml of 0.1 N NaOH), peroxide (50 mg in 50 ml of 3% peroxide), thermal (50 mg kept in air oven at 80 ^{0}C) and UV light (at 254 nm) degradation

studies. The % degradation and the number of degradation compounds formed in each degradation study were evaluated and the effectiveness of the method for the separation an analysis of degradation compounds of fluorometholone, neomycin and benzalkonium chloride was evaluated. The formulation solution was analysed in the developed method and the % assay of fluorometholone, neomycin and benzalkonium chloride was calculated [25-27].

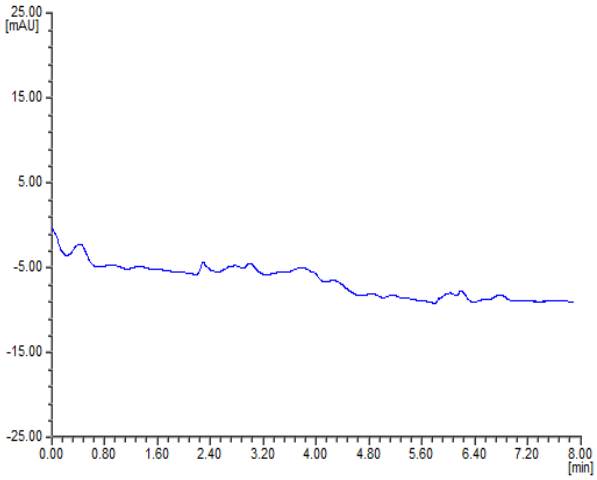
Results and Discussions:

The summary of the method developed for the separation and quantification of fluorometholone, neomycin and benzalkonium chloride in ophthalmic formulations was presented in table 1.

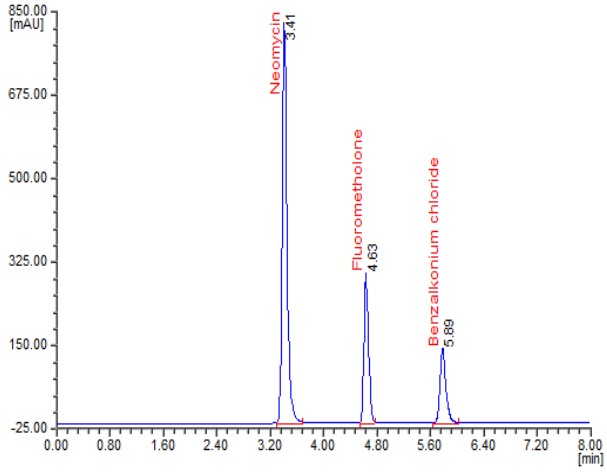
S. No	Mobile Phase composition	Result	Conclusion
1	MP: methanol, acetonitrile, 0.1% orthophosphoric acid in 55:40:05 (V/V); SP: Inertsil ODS 3V (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	No clear separation of analytes was observed and baseline was disturbed	Method Rejected
2	MP: methanol, acetonitrile, 0.1% orthophosphoric acid in 55:40:05 (V/V); SP: ProntoSIL ODS C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	Peaks corresponds to fluorometholone, neomycin and benzalkonium chloride was not separated and merged peaks was identified	Method Rejected
3	MP: methanol, acetonitrile, 0.1% orthophosphoric acid in 55:40:05 (V/V); SP: Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	Individual peaks were identified but the separation was acceptable and peak response was found to be very poor.	Method Rejected
4	MP: methanol and 0.1% aqueous formic acid in 65:35 (V/V); SP: Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	Peak corresponds to fluorometholone and neomycin was not resolved whereas the peak corresponds to benzalkonium chloride was resolved. The peak area response was noticed to be very less for all the analytes	Method Rejected
5	MP: acetonitrile and 0.1% aqueous formic acid in 65:35 (V/V); SP: Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	The peaks were not resolved and baseline was disturbed. The peak area response was noticed to be very less for all the analytes	Method Rejected
6	MP: acetonitrile and ammonium formate in 0.1% aqueous formic acid in 85:25 (V/V); SP: Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	Three peaks corresponds to analytes was observed. Peaks were observed to be little broad with tail factor.	Method Rejected
7	MP: acetonitrile and ammonium formate in 0.1% aqueous formic acid in 65:35 (V/V); SP: Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm); WL: 237 nm; FR: 0.6 mL/min	Symmetric peaks with acceptable peak shape and acceptable system suitability was observed.	Method Accepted

Table 1: Method development conditions tried during optimization process.

The separation of fluorometholone, neomycin and benzalkonium chloride was achieved on Spherisorb ODS 2 C18 (150 mm × 4.6 mm, 5 µm) column as stationary phase, acetonitrile and ammonium formate in 0.1% aqueous formic acid in the ratio of 65:35 (V/V) at pH 5.2 as mobile phase. The mobile phase was pumped in isocratic mode at a flow rate of 0.6 mL/min. The separated compounds were detected using UV detector at a wavelength of 237 nm. In the optimised condition, the chromatogram observed for blank and standard was given in figure 2a and 2b respectively.



2A) Black chromatogram



2B) Optimised chromatogram

Figure 2: System suitability chromatograms of fluorometholone, neomycin and benzalkonium chloride in the developed method

The calibration curve in the developed method was constructed from 25 % to 150 5 levels of the analytes. The linear calibration curve was observed within the concentration range of 2.5 – 15 µg/mL for fluorometholone, 8.75 – 52.5 µg/mL for neomycin and 0.1 – 0.6 µg/mL for benzalkonium chloride. The regression equation was found to be y = 30149x + 22236 (R^2 = 0.9996), y = 17221x + 54150 (R^2 = 0.9994) and y = 215856x + 6458.5 (R^2 = 0.9996) for fluorometholone, neomycin and benzalkonium chloride in the developed method. The linearity results observed in the developed method for the analysis of fluorometholone, neomycin and benzalkonium chloride was given in table 2.

S. No	Fluorometholone		Neomycin		Benzalkonium chloride	
	Con*	Peak Area	Con*	Peak Area	Con*	Peak Area
1	2.5	95358.7	8.75	213528.9	0.1	28946.1
2	5.0	175935.0	17.5	351062.7	0.2	49265.9
3	7.5	250861.8	26.25	499081.5	0.3	70959.6
4	10.0	319480.3	35.0	651159.3	0.4	91774.5
5	12.5	399457.1	43.75	814849.3	0.5	114150.9
6	15.0	475121.8	52.5	959653.4	0.6	136951.3

*Con = concentration of analyte in µg/mL

Table 2: Linearity results

The system suitability parameters like number of theoretical plates (plate count), tail (asymmetric) factor, resolution factor, retention time and relative retention time was calculated in the developed method and the results were found to be within the acceptable limit for fluorometholone, neomycin and benzalkonium chloride confirms that the method is suitable for the analysis.

S No	Analyte	Results achieved in the developed method for		
		Fluorometholone	Neomycin	Benzalkonium chloride
1	Test concentration (µg/mL)	10	35	0.4
2	Peak area response	319480.3	651159.3	91774.5
3	Retention Time (min)	4.63	3.41	5.89
4	Theo plates	6870	4138	8951
5	Tail Factor	0.95	0.98	1.03
6	Resolution	8.19	--	7.58

Table 3: System suitability results

The 100% standard solution containing 10 µg/mL of fluorometholone, 35 µg/mL of neomycin and 0.4 µg/mL of benzalkonium chloride was analysis in the developed method for the evaluation of precision (repeatability) and ruggedness (reputability) of the developed method. The % RSD in the peak area response was found to be 0.41, 0.36 and 0.29 in intraday precision, 0.42, 0.38 and 0.74 in interday precision and 1.03, 0.46 and 1.69 in ruggedness respectively for fluorometholone, neomycin and benzalkonium chloride. This confirms that the method is rugged and precise.

The robustness of the method was evaluated by analysing the 100% standard solution containing 10 µg/mL of fluorometholone, 35 µg/mL of neomycin and 0.4 µg/mL of benzalkonium chloride in the developed method will small variations. The % RSD of the peak area response was found to be with the acceptable limit of less than 2 for fluorometholone, neomycin and benzalkonium chloride. The system suitability conditions were also evaluated for

fluorometholone, neomycin and benzalkonium chloride in all the changed conditions and results found that there no considerable change in the results observed (table 4) confirms that the method is robust.

S No	Condition changed	Fluorometholone		Neomycin		Benzalkonium chloride	
		Area	% change	Area	% change	Area	% change
1	MP 1	316273.7	1.00	657042.6	0.90	92463.2	0.75
2	MP 2	318859.9	0.19	655251.3	0.63	91934.4	0.17
3	pH 1	318555.2	0.29	656377.1	0.80	91961.5	0.20
4	pH 2	316529.9	0.92	649540.5	0.25	92634.6	0.94
5	WL 1	320479.4	0.31	647998.9	0.49	91315.5	0.50
6	WL 2	317339.6	0.67	655232.4	0.63	91137.0	0.69

MP (mobile phase) 1: acetonitrile and ammonium formate in 0.1% aqueous formic acid in the ratio of 60:40 (V/V); MP 2: 70:30 (V/V); pH 1: 5.1; pH 2: 5.3; WL (wavelength) 1: 232 nm; WL 2: 242 nm

Table 4: Robustness results

Accuracy/recovery of the method was performed by following spiked recovery at 50%, 100 % and 150 % spiked levels of fluorometholone, neomycin and benzalkonium chloride. An acceptable % recovery in each analysis, % RSD in each spiked level was observed (table 5) fluorometholone, neomycin and benzalkonium chloride confirms that method is accurate.

S. No.	Compound	Recovery Level	Concentration in µg/mL	Amount found* Mean ± SD	% recovered* Mean ± SD	% RSD of Recovery
1	Fluorometholone	50 %	5	4.93±0.018	98.57±0.351	0.36
2		100 %	10	9.88±0.047	98.85±0.466	0.47
3		150 %	15	14.92±0.053	99.44±0.356	0.36
4	Neomycin	50 %	17.5	17.22±0.046	98.41±0.263	0.27
5		100 %	35	34.59±0.147	98.83±0.419	0.42
6		150 %	52.5	51.84±0.050	98.75±0.095	0.10
7	Benzalkonium chloride	50 %	0.2	0.20±0.001	98.57±0.351	0.44
8		100 %	0.4	0.40±0.003	98.85±0.466	0.66
9		150 %	0.6	0.59±0.005	99.44±0.356	0.89

* n=3

Table 5: Recovery results

The method sensitivity was evaluated by assessing the detection limit (LOD) and quantification limit (LOQ) analytes in the developed method. The limit of detection was found to be 0.313, 1.094 and 0.013 µg/mL whereas the LOQ was calculated as 1.031, 3.609 and 0.041 µg/mL respectively for fluorometholone, neomycin and benzalkonium chloride. This proved that the method was enough sensitivity for the detection of analytes in samples. The stability of analytes after stress exposer as well as the effectiveness of the method for the separation of degradation products (DPs) along with analytes was evaluated by conducting stress degradation studies. The very low % degradation of 2.61, 3.66 and 4.25 was for fluorometholone, neomycin and benzalkonium chloride respectively and two degradation products (DP 1 and DP 2) were well resolved and retained in the chromatogram (Figure 3). In UV light degradation study, a very high % degradation of 8.18, 7.74 and 6.69 % was observed respectively for fluorometholone, neomycin and benzalkonium chloride. In this five degradation products were well resolved from analytes (Figure 4). In acid degradation study, the % degradation was found to be 3.22, 4.38 and 7.91 for fluorometholone, neomycin and benzalkonium chloride respectively and the chromatogram clearly shows well resolved and retained four degradation compounds (Figure 5). The chromatograms observed in base (Figure 6) and thermal (Figure 7) degradation study clearly shows four and three degradation products. The % degradation in these studies was calculated to be less than the acid stress. In all the stress degradation conditions, the studied analytes viz., fluorometholone, neomycin and benzalkonium chloride were identified and retained same as the unstressed standard. This confirms that the method is effectively separates the degradation compounds formed during the stress study hence the method is stability indicating.

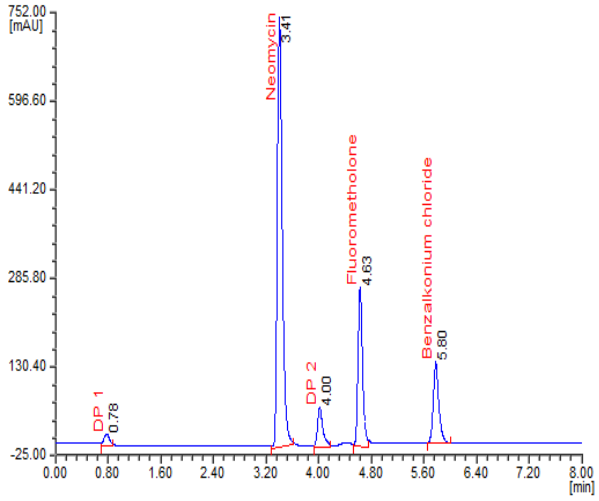


Figure 3: Peroxide degradation chromatogram

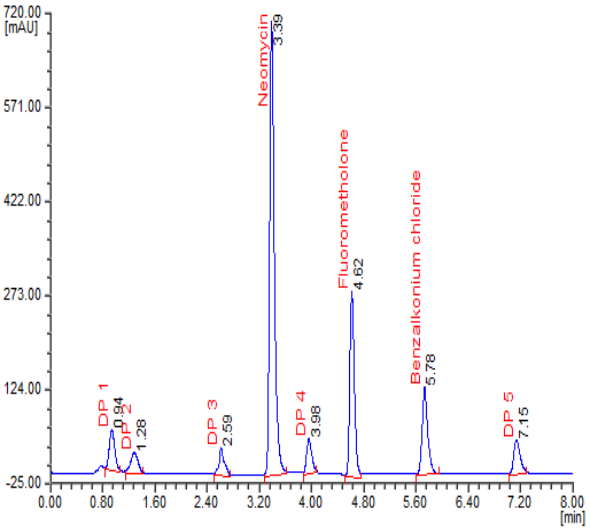


Figure 4: UV light degradation chromatogram

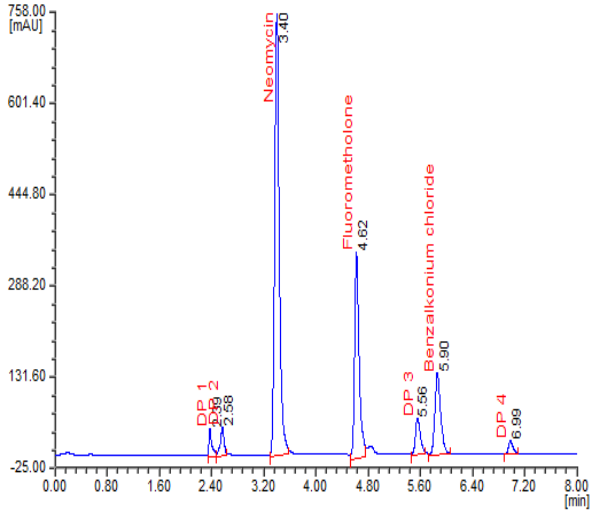


Figure 5: Acid degradation chromatogram

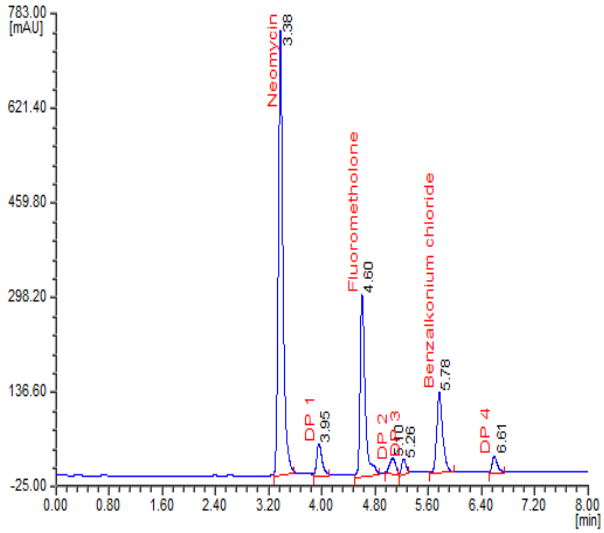


Figure 6: Base degradation chromatogram

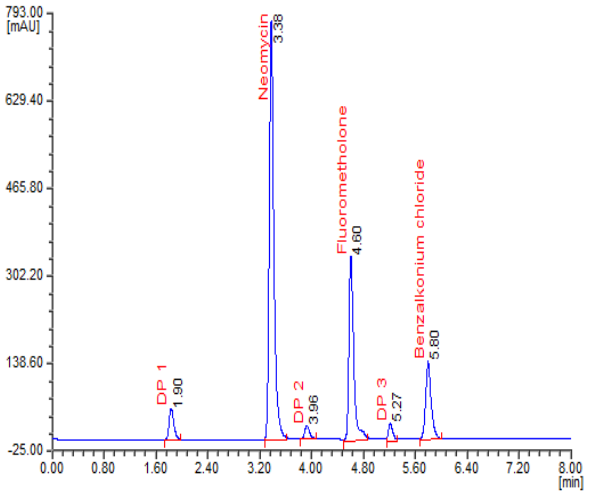


Figure 7: Thermal degradation chromatogram

The developed method is applied for the estimation of fluorometholone, neomycin and benzalkonium chloride in pharmaceutical eye drop formulations. The % assay in formulation sample was observed to be 99.05 %, 98.63 and 98.75 % for fluorometholone, neomycin and benzalkonium chloride respectively. In the formulation chromatogram [Figure 8], there is no formulation excipients were detected and the retention time of fluorometholone, neomycin and benzalkonium chloride were found to be very similar to the standard proves that the method is applicable for the routine analysis of fluorometholone, neomycin and benzalkonium chloride in formulations.

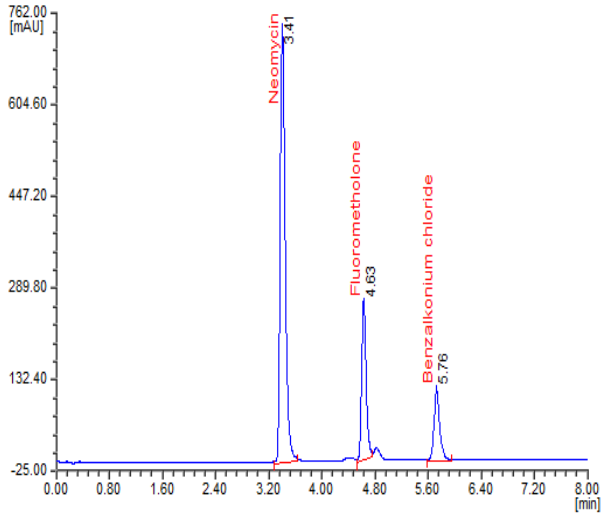


Figure 8: Formulation chromatogram

Conclusion:

The analytical RP-HPLC method satisfies all the method validation parameters such as specificity, system suitability, accuracy, linearity of detector response, precision, ruggedness (change in two different analysts) and robustness (variation in mobile phase composition, flow rate and pH). Meanwhile the method satisfactorily separates the unknown degradation compounds formed during forced degradation study. It confirms that the method is more stable and suitable for the analysis of fluorometholone, neomycin and benzalkonium chloride. Based on the findings achieved in the study, it can be concluded that, the method can be used for the routine analysis of fluorometholone, neomycin and benzalkonium chloride in eye drop formulations.

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