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A NEW STEBILITY INDICATING RP-HPLC-PDA METHOD FOR SIMULTANEOUS ESTIMATION OF NEOMYCIN AND FLUOCINOLONE IN PHARMACEUTICAL TABLET DOSAGE FORM

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Keywords:

RP-HPLC method development, Validation, Neomycin, Fluocinolone

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ABSTRACT: A simple, selective, ingenuous, reasonable, speedy, Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was developed for simultaneous estimation of neomycin and fluocinolone in its tablet dosage form. The separation was eluted using a mobile phase of buffer and methanol in the proportion of 50:50 a pumped at a flow rate of 1 ml/min besides 250 nm as a UV detection wavelength. The stationary phase used was column discovery 250 × 4.6 mm, 5µ. The drug samples were eluted at a retention time of neomycin 2.403 min and fluocinolone 3.303 min. The proposed analysis was developed and validated based on ICH guidelines by taking into account the parameters such as precision, accuracy, linearity, specificity, robustness, limit of detection, limit of quantification and degradation studies. The developed RP-HPLC method proved to be stability indicating by the resolution of samples with their forced degradation studies. The designed method used for routine analysis of neomycin and fluocinolone in combined dosage form. For peak detection and purity confirmation PDA was used as a tool.

INTRODUCTION: The component of neomycin produced from *Streptomycin fradiae*. On hydrolysis it yields neoamine and neobiosamine B. Neomycin is a bactericidal aminoglycoside antibiotic that binds to the 30s ribosome of susceptible organisms. Binding interferes with mRNA binding and acceptor tRNA sites and results in the production of non-functional or toxic peptides [1, 2, 3]. The neomycin is a variable mixture of two stereo isomers, neomycin B and C which are active components and neomycin A is degradation product [4, 5].

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The chemical name of neomycin is (2S, 3S, 4R, 5R, 6R) -5-amino-2-(aminomethyl)-6-{[(2R, 3S, 4R, 5S)-5-{[(1R, 2R, 3S, 5R, 6S)-3,5-diamino-2-{[(2R, 3R, 4R, 5S, 6R)-3-amino-6-(aminomethyl)-4,5-dihydroxyoxan-2-yl] oxy} -6- hydroxycyclo hexyl]oxy}-4-hydroxy-2-(hydroxymethyl) oxolan-3-yl] oxy} oxane-3, 4-diol. Neomycin assay interferences from drugs, such as bacitracin and polymyxin B and inactive components, *e.g.* wax, were eliminated by a methanol wash and/or a partitioning method [6].

The extracted neomycin was derivatized with 2, 4-dinitrofluorobenzene followed by normal-phase HPLC with detection at 254 nm. The average recovery of neomycin from spiked samples was ˜100% with a relative standard deviation of <1% [4]. Now these neomycin and fluocinolone are official in US pharmacopeia [7]. The structure of neomycin was shown below.

Fluocinolone is glucocorticoid derivative used topically in the treatment of various skin disorders and inflammatory eye, ear, and nose diseases [8]. It has high anti inflammatory activity and is usually used formulated as a cream, gel, lotion, or ointment [9, 10, 11]. It is usually employed as a cream, gel, lotion, or ointment. It has also been used topically in the treatment of inflammatory eye, ear and nose disorders [12]. The chemical name of fluocinolone is (1S, 2S, 4R, 8S, 9S, 11S, 12R, 13S, 19S)-12,19-difluoro-11-hydroxy-8-(2-hydroxyacetyl)-6,6,9,13-tetramethyl-5,7-dioxapentacyclo [10.8.0.0^{2}, ^{9}.0^{4}, ^{8}.0^{13}, ^{18}] icosa-14,17-dien-16-one [13, 14] and the was shown below. A few analytical methods have been reported for neomycin and fluocinolone individually or with some other combination; some of them were being development of an RP-HPLC method for analysis of fluocinolone acetonide in gel and ointment [9]. Analysis of residual neomycin in honey by LC-MS/MS [15]. There is no official method reported for this combination so far.

The main aim of this method was to determine and validate, in assimilation with International Conference on Harmonization guidelines [16, 17, 18, 19], an accurate, economical, and reproducible procedure for quantitative analysis of neomycin and fluocinolone in the bulk drug and in tablet dosage forms. It was thought advisable to develop precise, accurate, simple RP-HPLC method for simultaneous estimation of neomycin and fluocinolone.

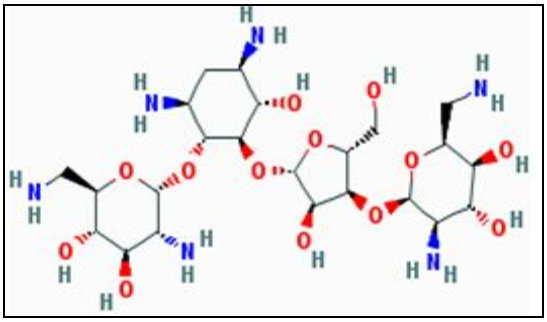

FIG. 1: STRUCTURE OF NEOMYCINE

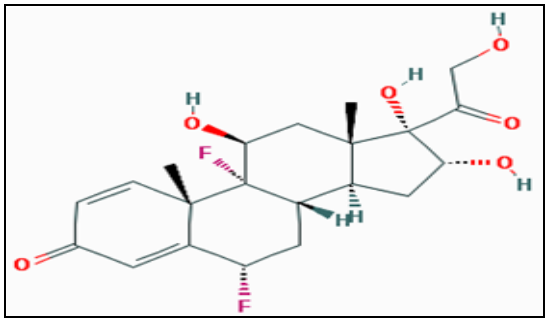

FIG. 2: STRUCTURE OF FLUOCINOLONE

MATERIAL AND METHODS:

Instrumentation: The HPLC instrument was used for the analysis of neomycin and fluocinolone is WATERS HPLC 2965 SYSTEM with auto injector. It has PDA detector which can determine the peak area and purity confirmation. The system was controlled by using software Empower 2 and it has UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absor-bance for neomycin and fluocinolone solutions. The quantization and separation were conducted on the column discovery 250 × 4.6 mm, 5µ.

Materials: The materials required for the analysis of neomycin and fluocinolone were HPLC grade phosphate buffer, ammonium acetate buffer, glacial acetic acid, methanol, potassium dihydrogen phosphate buffer, tetra hydro furan, tri ethyl amine, water, ortho phosphoric acid AR grade were secured from SD Fine Chem. Mumbai, India. The drug samples were compassionately provided by Spectrum Pharmaceuticals Pvt., Ltd., Kukutpally, Hyderabad, India.

The formulation samples for the current study was analytical grade of sodium hydroxide, hydrochloric acid, hydrogen peroxide and high purity distilled water were purchased from local market. The standards preparation of two drugs were made use of flutop tablets labelled claim was 5 mg of neomycin, 0.25 mg fluocinolone.

General Procedures:

Buffer: (0.1%OPA): 1 ml of ortho phosphoric acid solution in a 1000 ml of volumetric flask added about 100 ml of milli-Q water and final volume make up to 1000 ml with milli-Q water.

Sample Preparation: Accurately 20 tablets were weighed using Electronic balance [BL-220H, Shimadzu Corporation, Japan] and calculated the average weight of each tablet then the tablet powder weight equivalent to 5 mg of neomycin and 2.5 mg of fluocinolone was transferred into a 1000 ml volumetric flask, 100 ml of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution

1 ml was pipetted out into a 10 ml volumetric flask and made up to mark with diluent.

Mobile Phase: Buffer and methanol in the ratio 50:50A was used as the mobile phase

Standard Preparation: (50 µg/ml neomycin and 2.5 µg/ml fluocinolone). Accurately weighed using Electronic balance [BL-220H, Shimadzu Corporation, Japan] and transferred 5 mg and 2.5 mg of neomycin and fluocinolone working standards into 10 ml and 100 ml clean dry volumetric flasks separately, add 100 volume of diluent, sonicated for 30 min and make up to the final volume with diluents.

From the above each stock solution, 1 ml was pipetted out in to a 10 ml volumetric flask and then make up to the final volume with diluent. These concentrations were selected based on recommended guidelines by the International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) for analytical methods validation [16, 17, 18, 19].

Label Claim: 5 mg of neomycin + 0.25 mg of fluocinolone

Optimization of Chromatographic Conditions: A mobile phase system consisting of 0.01M ortho-phosphoric acid and methanol was used in the ratio of 50:50% v/v at a pH 2.5 adjusted with ortho phosphoric acid and it is also used as diluents for preparing the working solution of drugs. The separation was performed with the elution method and flow rate was 1.0 ml/min. The injection volume was 10 µL. The eluant was proctor by the photo diode array detector (PDA) from 200 to 400 nm, and chromatograms were gained at the wavelengths of 250 nm. The total run time was 7 min and all establishments were performed at 30 ºC.

Forced Degradation Studies: Oxidation: To 1 ml of stock solution of neomycin, fluocinolone and 1 ml of 20% hydrogen peroxide (H_2O_2) were added separately and these solutions were kept for 30 min at 60 ºC. The resultant solution was diluted to obtain concentrations 50 µl, 2.5 µl. for neomycin and fluocinolone respectively. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies: To 1 ml of stock solution of neomycin, fluocinolone and 1ml of 2N hydrochloric acid was added and refluxed for 30 min at 60 ºC. The resultant solution was diluted to obtain concentrations 50 µl, 2.5 µl for neomycin and fluocinolone respectively. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies: To 1 ml of stock solution of neomycin, fluocinolone and 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 ºC. The resultant solution was diluted to obtain concentrations 50 µl, 2.5 µl for neomycin and fluocinolone respectively. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard stock solution was kept in oven at 105 ºC for 6 h to study dry heat degradation. The resultant solution was diluted to obtain concentrations 50 µl, 2.5 µl for neomycin and fluocinolone respectively for HPLC study. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability Studies: The photochemical stability of the drugs were also examined by exposing the concentrations of 50 µl, 2.5 µl solution to UV Light by placing the beaker in UV Chamber for 7 days or 200 watt hours/m^2 in photo stability chamber. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the standard solutions in water for 6 h at a temperature of 60 ºC. A solution of 10 µl was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Method Development: Three trials were executed for the method development and the best peaks with least fronting factor was elevated for neomycin and fluocinolone with RT = 2.403 min, RT = 3.304 min. accordingly. The resultant chromatogram is shown in the **Fig. 3**.

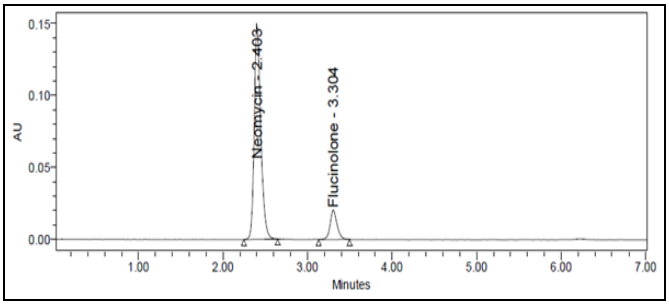

FIG. 3: CHROMATOGRAM OF NEOMYCIN AND FLUOCINOLONE

Method Validation: The method was validated according to ICH guidelines [16, 17, 18, 19]. The different validation characteristics which were determined are the following: system suitability test, precision, accuracy, linearity and specificity, limit of detection, limit of quantification, robustness, degradation studies and the stability indicating capability.

System Suitability Test: Six replicate injections of standard solution were injected and the chromatograms were recorded. The system was suitable for analysis if the % relative standard deviation (%RSD) of area counts in six replicate injections should be not more than 2.0%. USP tailing factor for neomycin and Fluocinolone peak should be not more than 2.0. USP resolution factor between the peaks corresponding to neomycin and fluocinolone should be more than 2.0. The results are revealed in **Table 1**.

TABLE 1: SYSTEM SUITABILITY PARAMETERS

Parameters	Neomycin	Fluocinolone
Tailing Factor	1.28	1.12
Theoretical plates	4830	8246
USP Resolution	--	6.1
LOD(µg/ml)	0.02	0.01
LOQ(µg/ml)	0.06	0.02

Precision: The standard neomycin and fluocinolone solutions were injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six repeat injections was established to be within the specific limits. The data was presented in the **Table 2**.

Acceptance Criteria: The % RSD should not be more than 2%.

TABLE 2: PRECISION STUDY

S. no.	Neomycin			Fluocinolone		
	Peak area	% Assay	Day-day precision	Peak area	% Assay	Day-day precision
1	800603	99.89	640659	115822	98.76	91754
2	807258	100.72	643598	116695	99.50	91666
3	807456	100.74	636404	117591	100.26	92757
4	800729	99.91	639740	115123	98.16	92829
5	809126	100.95	634729	116323	99.01	94731
6	800552	99.88	641253	115789	98.73	93015
AVG	804287	100.35	639397	116191	99.07	92792
SD	4061.2	0.51	3272.7	855.2	0.7	1109.7
%RSD	0.5	0.50	0.50	0.7	0.7	1.2

Accuracy: Injected the standard solutions of accuracy 25%, 50% and 75% for neomycin, 1.25%, 2.5%, 3.75% for fluocinolone and calculated the amount found, Amount added for neomycin and fluocinolone and the individual recovery and mean recovery values are shown in the **Table 3**.

TABLE 3: ACCURACY DATA

Sample	Neomycin			Fluocinolone		
% Concentration	25%	50%	75%	1.25%	2.5%	3.75%
Trail-I	100.86	99.14	98.36	100.19	98.91	100.16
Trail-II	99.93	98.72	99.07	98.17	99.89	100.57
Trail-III	99.05	98.92	98.93	100.28	98.06	99.51
AVG (%Recovery)	99.95	98.93	98.79	99.54	98.95	100.05
SD	0.902540	0.211438	0.378347	1.19	0.918328	0.586636
%RSD	0.90	0.21	0.38	1.20	0.93	0.59

Acceptance Criteria: The % recovery for neomycin and fluocinolone at each level should be between 99 to 101%.

Recovery Studies: To determine the accuracy and precision of the proposed method recovery studies were carried out.

A fixed amount of sample was taken and reference drugs were added at 25%, 50% and 75% for neomycin, 1.25%, 2.5%, 3.75% for fluocinolone. The results were analyzed and found within the limits.

Linearity and Calibration Curve: Working dilutions of neomycin and fluocinolone in the range of 12.5 - 75 and 0.625 - 75% were prepared by considering appropriate aliquots of functioning standard solutions of drugs in various 10 ml volumetric flask and diluting up to the mark with mobile phase. 10 µl quantity of every dilution was injected in to the column at a flow rate of 1ml/min. The drug in elute was monitored at 250 nm and the resultant chromatograms were recorded. From these, the mean peak areas were computed and shown in the **Table 4**. A plot of concentration vs. peak areas was constructed and shown in the **Fig. 4** and **5** for neomycin and fluocinolone respectively.

The regression of the plot was calculated by least square regression method. The slope and intercept value for calibration curve for neomycin and fluocinolone was y=15363x+7213. (R^2=0.999) and y=39413x+334.4 (R^2=0.999) respectively.

TABLE 4: LINEARITY MEANS PEAK AREA VALUES

S. no	Concentration of neomycin (µg/ml)	Response	Concentration of fluocinolone (µg/ml)	Response
1	0	0	0	0
2	12.5	210475	0.625	24421
3	25	389897	1.25	48121
4	37.5	574831	1.875	76074
5	50	791354	2.5	101215
6	62.5	953690	3.125	123953
7	75	1163070	3.75	145859

*Each response value is a mean of three readings

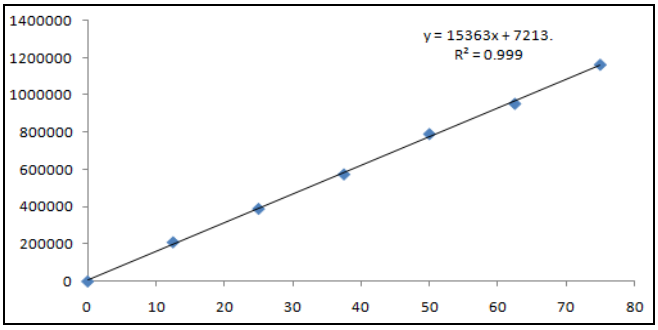

FIG. 4: LINEARITY GRAPH FOR NEOMYCIN

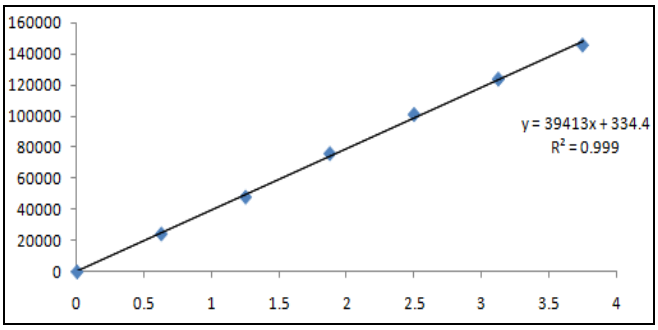

FIG. 5: LINEARITY GRAPH FOR FLUOCINOLONE

X-Axis = Concentration, Y-Axis = Peak area

Specificity: The specificity of the HPLC method is provided, where complete separations of neomycin and fluocinolone were detected in presence of other inactive excipients used in tablets.

TABLE 5: SPECIFICITY STUDY

S. no.	Name	No. of Injections	Neomycin Area	Fluocinolone Area
1	Blank	1	-	-
2	Placebo	1	-	-
3	Standard	1	799949	116672
4	Sample	1	799887	117046

In addition, there was no any deterrence at the retention time in the chromatogram of placebo solution. In peak purity analysis with PDA, purity angle was always less than purity threshold for the analyte. This shows that the peaks of analyte were pure and excipients in the formulation does not interfere the analyte. The data are presented in the **Table 5**.

Limit of Detection and Limit of Quantification: Limit of Detection (LOD) is the lowest concentration of an analyte in a sample that can be detected but not quantified. LOD is expressed as a concentration at a specified signal to noise ratio. The LOD will not only depend on the procedure of analysis but also on the type of instrument. In chromatography, detection limit is the injected amount that results in a peak with a height at least twice or thrice as high as baseline noise level.

The LOD for neomycin was found to be 0.02.
The LOD for fluocinolone was found to be 0.01.

Limit of Quantification (LOQ) is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. LOQ is expressed as a concentration at a specified signal to noise ratio. In chromatography, limit of quantification is the injected amount that results in a peak with a height, ten times as high as base line noise level.

The LOQ for neomycin was found to be 0.06.
The LOQ for fluocinolone was found to be 0.02.

Robustness: Robustness is estimated by making deliberate changes in the chromatographic conditions like change in temperature, flow rate, and mobile phase composition and evaluated for the impact on the method. It was observed from the chromatograms that the results were within the limits. This indicates that the method developed is robust and the data was shown in the **Table 6**.

TABLE 6: ROBUSTNESS STUDY

Parameter		Neomycin	Fluocinolone
Temperature	25 ºC	636543	91740
± 5 ºC	35 ºC	669823	95754
Flow rate	0.9 ml	895410	127632
± 0.1ml	1.1 ml	560579	82492
Mobile phase change	45:55	718119	106278
± 5 pH	55:45	67047	96266

Degradation Studies: The stress studies were performed to make sure the efficient separation of neomycin and fluocinolone in the present analysis from degradation products. The degradation was observed by standard peak areas of the drug substances with same drug molecules of degraded peak areas. The percentage of degradation was designed from the peak area acquired in degradation conditions and it was compared with assay of standard conditions.

TABLE 7: DEGRADATION STUDIES OF NEOMYCIN AND FLUOCINOLONE

Sample name	Total purity	Neomycin			Fluocinolone		
		% of Purity	% of Degradation	Purity of peak area	%of Purity	% of Degradation	Purity of peak area
Acid	100	95.07	4.93	1072054	95.01	4.99	1487557
Base	100	97.30	2.70	1095361	97.49	2.51	1508377
Peroxide	100	98.68	1.32	1083949	98.43	1.57	1517149
Thermal	100	99.28	0.72	1105245	99.48	0.52	1502279
UV	100	99.25	0.75	1118510	99.25	0.75	1511176
Water	100	99.36	0.64	1148042	99.45	0.55	1559554

The percentage assay degradation in both acidic and alkali conditions was found to be within the limits. Oxidative degradation studies, thermal, photo, neutral stability studies were conducted by applying the conditions mentioned in the general procedures. The purity of angle is found to be less than that of purity of threshold in all the conditions which indicates that the developed method was stability indicating. The forced degradation studies were conducted without planning to identify the degradation products but only to show that they are not interfering with active molecules if any present. The data of stress studies are shown in **Table 7**.

RESULTS AND DISCUSSION: A simple, rapid, economical, precise and accurate method has been developed and validated for the drug neomycin and fluocinolone.

TABLE 8: RESULTS OF ALL PARAMETERS

S. no.	Parameter	Acceptance criteria	Observed value	
			Neomycin	Fluocinolone
1	Accuracy	98-101%	99.22	99.51
2	Precision	RSD within 2%	0.50	0.7
3	Linearity	R^2 not less than 0.999	R^2=0.9993	R^2=0.999
4	LOD	S/N=3	0.02	0.01
5	LOQ	S/N=10	0.06	0.02

The estimation was carried out with a mixture of buffer and methanol at pH 2.5 adjusted with ortho phosphoric acid in the ratio of 50:50% v/v. Precision of the methods were studied by making repeated injections of the samples and system precision values were determined.

The retention time was 2.403 min and 3.303 min. The calibration curve was linear over the concentration range of 12.5-75 µl for neomycin and 0.625-3.75. The LOD values were 0.02, 0.01 and LOQ values were found to be 0.06, 0.2 The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method and the forced degradation studies shows that the developed method was stability indicating.

Hence it was concluded that the RP-HPLC method developed was highly suitable for routine analysis of pharmaceutical samples and all the parameters result data are shown in the below **Table 8**.

CONCLUSION: The current study describes new and simple RP-HPLC method for the simultaneous estimation of neomycin and fluocinolone. The method validated was found to be simple, accurate economical and precise. Therefore, the planned method can be used for quantification of neomycin and fluocinolone in bulk and pharmaceutical dosage form. Finally, this method was vigilantly validated; as a result, it can be recommended for routine analysis and for testing quality during stability studies of the drugs.

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CONFLICT OF INTEREST: The authors are declaring that there is no conflict of interest.

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