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Stability indicating method development and validation for the simultaneous estimation of Fluorometholone and Neomycin in bulk and Pharmaceutical dosage forms by RP-HPLC

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ABSTRACT

A Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method has been developed and validated for simultaneous estimation of Fluorometholone and Neomycin in bulk and pharmaceutical formulations. Separation was carried out using column Enable C18 (250 mm x 4.6mm x 5µm particle size) in isocratic mode using mobile phase composition Acetonitrile and 0.1% Orthophosphoric acid (pH 3.5) in the ratio 70:30 v/v and UV detection at 239 nm. The compounds were eluted at a flow rate of 0.8 ml/ min. The average retention times for Fluorometholone and Neomycin were 4.336 and 3.770 min, respectively. The method was validated according to the ICH guidelines. The % RSD of all validation parameters found to be less than 2% indicating high degree of accuracy and precision of the proposed HPLC method. The method was linear over the concentration of 10-50 µg/ml and 20- 100 µg/ml for Fluorometholone and Neomycin respectively. The LOD and LOQ of Fluorometholone were found to be 0.625 µg/ml and 1.8950 µg/ml and of Neomycin were found to be 1.0142 µg/ml and 3.0735 µg/ml.

Keywords: Fluorometholone, Neomycin, RP-HPLC, Validation and ICH guidelines.

INTRODUCTION

High Performance Liquid Chromatography is a special branch of column chromatography used to separate compounds that are dissolved in solution. HPLC is characterized by the use of high pressure to push a mobile phase solution through a column of stationary phase allowing separation of complex mixtures with high resolution.

Fluorometholone is a synthetic glucocorticoid and it is used for the treatment of allergic and inflammatory eye conditions. It is chemically known as (1R,2S,8S,10S,11S,14S,15S,17S)-14 acetyl-1-fluoro-14,17-dihydroxy-2,8,15-trimethyl tetracyclo [8.7.0,0,] [2, 7, 11, 15] heptadeca-3,6-dien-5-one. It occurs as yellow to off-white solid and is soluble in ethanol, methanol and insoluble

in water. It acts by the induction of phospholipase A2 inhibitory proteins, collectively called as lipocortin's. These lipocortin's controls the biosynthesis of mediators in inflammation, especially prostaglandins and leukotrienes, via inhibiting the release of the precursor molecule arachidonic acid [2-4].

Neomycin is an aminoglycoside antibiotic and it is used for topical infection treatment. It is chemically known as (2R,3S,4R,5R,6R)-5-amino-2 (aminomethyl)-6-[(1R,2R,3S,4R,6S)-4,6-diamino-2-[(2S,3R,4S,5R)-4-[(2R,3R,4R,5S,6S)-3 amino-6-(aminomethyl)-4,5-dihydroxyoxan-2-yl]oxy-3-hydroxy-5

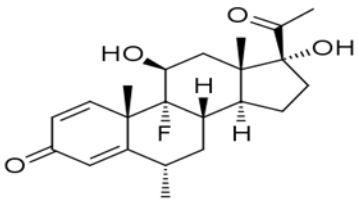

Fig.1: Structure of Fluorometholone

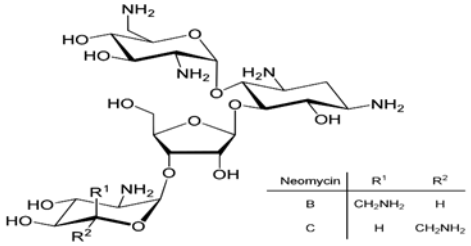

Fig.2: Structure of Neomycin

(hydroxymethyl)oxolan-2-yl]oxy-3-hydroxy-3hydroxy cyclohexyl] oxyoxane-3,4-diol. It occurs as white to yellowish powder and soluble in water and ethanol. Neomycin is bactericidal in action. Similar to other aminoglycosides, it inhibits bacterial protein synthesis through irreversible binding to 30 S ribosomal subunit of susceptible bacteria. Neomycin is actively transported into the bacterial cell where it binds to receptors present on the 30 S ribosomal subunit. This binding interferes with the initiation complex between messenger RNA (mRNA) formed. Eventually bacteria will die because of the lack of functional proteins [5-7].

The combination of Fluorometholone and Neomycin is used for treatment of bacterial eye infection, conjunctivitis and inflammation of eyes.

Literature survey reveals that there are several methods are reported for the estimation of Fluorometholone and Neomycin individually and in combination with the other drugs. But no method has been reported for the simultaneous estimation of these two drugs in combined formulations [8-12]. The present work describes the development of stability indicating RPHPLC method, which can quantify these components simultaneously from a combined dosage form. The present RP-HPLC method was validated according to (ICH) guidelines [13-14].

MATERIALS AND METHODS

Experimental

Instruments and columns

Shimadzu with high pressure liquid chromatographic instrument provided with a LC-20AD Pump and Prominence SPD 20AD UV-detector. Shimadzu-1800 UV-Vis Spectrophotometer was used with 1 cm match quartz cell of 10 mm optical path length, spectral band width of 1 ± 0.2 nm, and wavelength accuracy of ± 0.3 nm. The column used in the development for determination is Enable C18 (250 mm x 4.6 mm; 5µm).

Chemicals used

- Distilled water (HPLC grade).
- Acetonitrile (HPLC grade).
- Orthophosphoric acid (AR grade).

Drug samples used

Standard Fluorometholone was obtained as gift samples from Micro labs Ltd, Bangalore. Standard Neomycin was procured from Brawn Laboratories Ltd, Faridabad, Haryana.

Selection of analytical wavelength

Effect of wavelength on the response factor and on the peak parameters (asymmetry, tailing factor, resolution, theoretical plates) was studied over the

wavelength range of 400-200 nm. Satisfactory chromatographic conditions were obtained with a wavelength of 239.0 nm for Multicomponent analysis using HPLC method.

Preparation of mobile phase

A mixture of HPLC grade Acetonitrile and 0.1% orthophosphoric buffer in the ratio of 70:30 v/v was prepared and pH was adjusted to 3.5 and filtered through 0.45 µm membrane filter paper and sonicated for 20mins.

Preparation of standard stock solution

100 mg each of FLUO and NEOM were weighed separately and transferred into two different 100 ml volumetric flasks. Both the drugs were dissolved in 50 ml of mobile phase by sonication and then volume was made up to the mark with mobile phase to get a concentration of 1000 µg/ml of each component (stock A and A' solution).

From the above stock A and A' solution 10 ml of aliquot was pipetted out into a 100ml volumetric flask and the volume was made up to the mark with mobile phase to obtain a concentration of 100 µg/ml of each component (stock B and B' solution).

Analysis of formulation

Commercially available formulation FML NEO was purchased and 6 containers of the formulation was taken. From this formulation, 100mg of drug equivalent to Neomycin was taken which also contains 28.58mg of Fluorometholone and transferred to 100 ml volumetric flask, dissolved in sufficient quantity of mobile phase. The solution was filtered through 0.4 µm membrane filter paper. The contents were sonicated for 20 minutes and the final volume was made up to the mark with mobile phase to get the concentration of 1000 µg/ml of NEOM and this solution was used as stock "A" solution of the sample.

From the above stock "A" solution, 10ml of the aliquot was pipetted out and transferred to a 100ml volumetric flask. The volume was made up to 100 ml with mobile phase to obtain a concentration of 100 µg/ml of NEOM (stock "B" solution of the sample).

Appropriate aliquots were pipetted out from the sample stock "B" solution (100 µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range of 20, 40, 60, 80 and 100 µg/ml of NEOM and 10, 20, 30, 40 and 50 µg/ml of FLUO.

A 20 µl volume of each sample mixture was injected in to the sample injector of HPLC system and their chromatograms were recorded under the same chromatographic conditions as described above. The area of each peak was determined at 239.0 nm and the amount of drug present in the sample mixture was determined.

HPLC conditions

Table.1: Chromatographic conditions

Instrument	**High Performance Liquid Chromatography SHIMADZU – SPD – 20AD Detector**
Injector	Rheodyne 20 µl
Column	Enable C_{18} (250*4.5*5)
Wavelength	UV Detector
Detector	239 nm
Flow rate	0.8 ml/min
Injection volume	20 µl
Mobile phase	Acetonitrile and 0.1 % OPA buffer 3.5 (70:30 v/v)

Method validation

Validation of the optimized method was performed according to the ICH guidelines.

Linearity and range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. The range of analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

For the linearity 100% each of working standard solution of FLUO and NEOM were injected at Concentration 10-50 µg/mL and 20-100 µg/mL respectively and the results obtained are tabulated as follows in the Table.2 and calibration curves are shown in Fig. 7 & 8. The results show an excellent correlation exists between mean peak area and concentrations level of drugs within the concentration range. The regression equation calculated by least square method was y =42846 x + 26907 and y = 11494x + 1378.1 with correlation coefficient of both drugs R^2 = 0.9995 and R^2 = 0.9998.

Accuracy

Recovery studies were carried out by adding 80%, 100% and 120% of the standard drug solution of FLUO and NEOM to the known amount of sample solution by standard addition method. The results obtained for recovery were found to be within the limits. The results are tabulated in Table.3.

Precision

It is the procedure which express closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

The Intraday and Inter-day precisions of the proposed HPLC method were determined by estimating the corresponding responses three times on the same day and on 3 different days over a period of one week for 3 different concentration and 3 replicates of FLUO and NEOM and reported in terms of relative standard deviation (RSD). Statistical validation of data for Intraday and Inter day precision methods as shown in Table.4.

Robustness

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters. The solution containing 30 µg/ml of FLUO and 60 µg/ml of NEOM was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate and wavelengths. The results are shown in Table.5

Ruggedness

The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in analyst or instrument. The results are given in Table.6.

LOD and LOQ

The limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The limit of quantitation (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

In this study, LOD and LOQ were determined based on the standard deviation of the response and slope of the corresponding curve using the following equations,

Where, $$\mathbf{LOD} = \frac{3.3\,\sigma}{S} \qquad \mathbf{LOQ} = \frac{10\,\sigma}{S}$$

σ is the standard deviation of the response
S is the slope of the calibration curve.

System suitability studies

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), a number of theoretical plates and tailing factor were evaluated for six replicate injections of the drug. The results which are given in Table.7 were within acceptable limits. System suitability test is a pharmacopeia requirement. Summary parameters of the developed methods shown in Table.7

RESULTS AND DISSCUSSION

The selected drugs Fluorometholone and Neomycin in Bulk and Formulation were estimated by RP-HPLC as per ICH guidelines. The methods were validated for all validation parameters as per ICH guidelines. The linearity range in both methods for FLUO and NEOM was 10-50 µg/ml and 20-100 µg/ml respectively. The % RSD for intraday and inter-day precision was found to be less than 2%. The accuracy of the methods was validated by recovery studies and was found to be significant and within specification limits, with % recovery 98-102%. The assay results were found to be within the acceptable limits

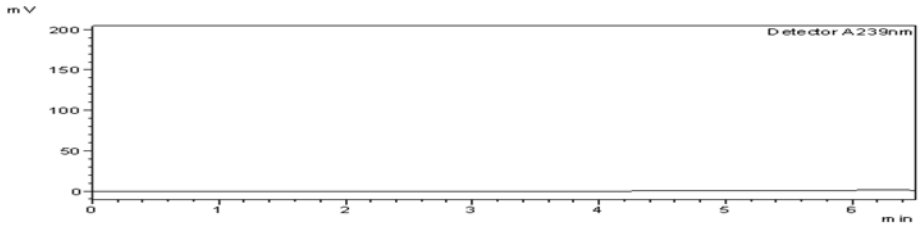

Fig.3: Chromatogram of Blank.

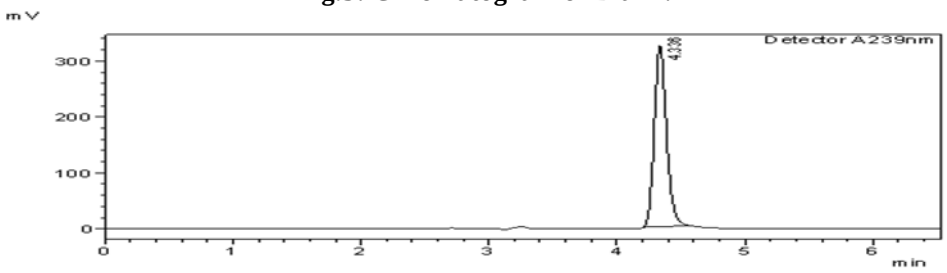

Fig.4: Chromatogram of FLUO at 239.0nm

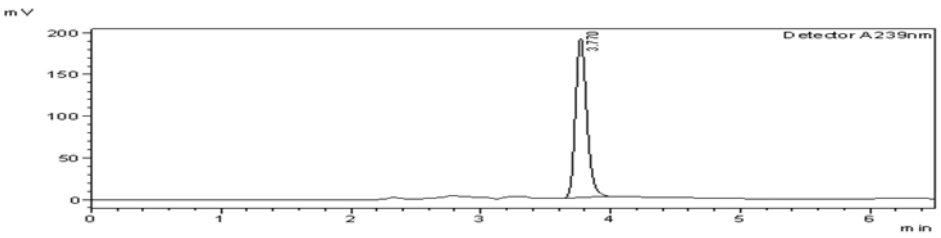

Fig.5: Chromatogram of NEOM at 239.0nm.

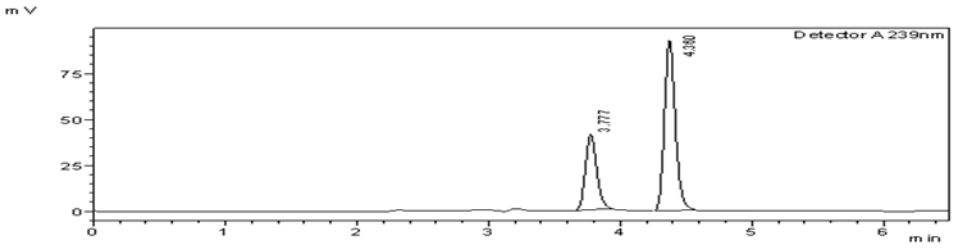

Fig.6: Chromatogram of FLUO and NEOM at 239.0nm.

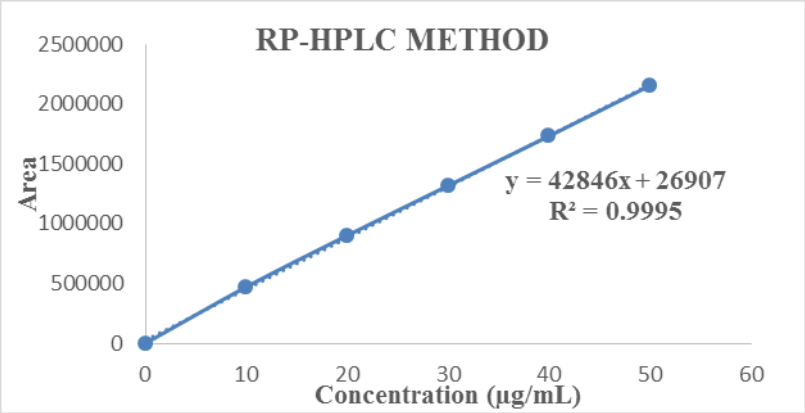

Fig.7: Calibration curve for FLUO at 239.0 nm by RP-HPLC method

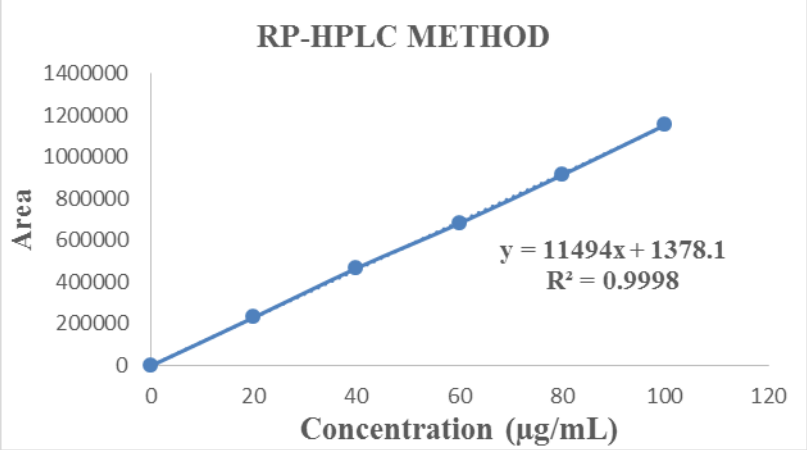

Fig.8: Calibration curve for NEOM at 239.0 nm by RP-HPLC method

Table.2: Linearity data of FLUO and NEOM

Sr. No.	FLUO		NEOM	
	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area
1	0	0	0	0
2	10	471998	20	232055
3	20	902976	40	468980
4	30	1319964	60	683458
5	40	1735982	80	916159
6	50	2157440	100	1155789

Table.3: Statistical Validation Data for Accuracy determination.

Level of % recovery	Mean*		Std. Deviation*		Co-efficient of Variation*		Standard Error*	
	FLUO	NEOM	FLUO	NEOM	FLUO	NEOM	FLUO	NEOM
80%	100.29	100.17	0.3614	0.1785	0.3604	0.1782	0.0178	0.0122
100%	100.2	99.99	0.3605	0.2428	0.3598	0.2428	0.0788	0.0233
120%	100.25	100.12	0.3094	0.1735	0.3086	0.1733	0.0011	0.0230

Table.4: Statistical Validation Data for Precision

Precision	Intra-day		Inter-day	
Components	**FLUO**	**NEOM**	**FLUO**	**NEOM**
Mean	100.12	99.98	100.19	100.04
Std Deviation	0.3356	0.2052	0.3549	0.1551
RSD	0.3351	0.2052	0.3542	0.1550
Std Error	0.0023	0.0043	0.0126	0.0223

Table.5: Data of Robustness.

Method Parameter	Level	Retention time		Tailing Factor	
		FLUO	**NEOM**	**FLUO**	**NEOM**
Flow rate (ml/min)	**0.7**	4.420	3.822	1.183	1.200
	0.8	4.336	3.770	1.171	1.180
	0.9	4.296	3.720	1.165	1.169
Wavelength (nm)	**-2**	4.296	3.712	1.165	1.167
	0	4.336	3.770	1.171	1.180
	-2	4.418	3.828	1.190	1.200

Table.6: Ruggedness result for variations in Analyst.

Method Parameter	Retention Time		Tailing Factor	
Analysts	**FLUO**	**NEOM**	**FLUO**	**NEOM**
Analyst 01	4.336	3.770	1.171	1.180
Analyst 02	4.360	3.787	1.186	1.172

Table.7: Summary of validation and System suitability parameters of Fluorometholone and Neomycin.

Parameters	FLUO	NEOM
Linearity Range µg/mL	10-50	20-100
Slope	42846	11494
Intercept	26907	1378.1
Regression Coefficient (r^2)	0.9995	0.9998
Limit of Detection (µg/mL)	0.6253	1.0142
Limit of Quantification (µg/mL)	1.8950	3.0735
Retention time (min)	4.336	3.770
Tailing factor	1.171	1.180
Resolution factor	3.283	

CONCULSION

The developed RP-HPLC method was found to be simple, precise, specific, and accurate and can be used for routine analysis of Fluorometholone and Neomycin. Both methods were validated as per ICH guidelines.

Acknowledgement

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