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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ANTIBIOTIC AND ANTI-INFLAMMATORY DRUGS IN OPHTHALMIC DOSAGE FORM BY HPLC

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ABSTRACT

The main objective of the present is to develop simple, precise, accurate and reproducible UV spectrophotometric and stability indicating HPLC methods for estimation of gatifloxacin sesquihydrate (GATI) and fluorometholone acetate (FLM) in ophthalmic dosage form. Dual wavelength spectrophotometric method, which involves solving of simultaneous equations based on the measurement of absorbance at 290 nm and 240 nm, which are the absorption maxima (ʎmax) of GATI and FLM respectively. The HPLC analysis is carried out on Hypersil Gold C18 column (4.6 x 250 nm, 5µ), using phosphate buffer (pH 3) in acetonitrile and methyl alcohol in the ratio of (60:40 v/v) as the mobile phase with a flow rate of 1.0 ml/min. The detection was carried out at a wavelength of 274 nm. The retention time were found to be 4.730 ± 0.5 min and 12.974 ± 0.5 min for GATI and FLM respectively. The percentage recoveries of both the drugs GATI and FLM were 98.6-100.5 % and 98.9-100.4 % respectively. The linearity range were found to be 50-150% for both the drugs is <1, respectively. The value of standard deviation and % R.S.D were found to be <2%. The simultaneous estimation of GATI and FLM in ophthalmic dosage form by HPLC method were estimated and validated according to ICH guidelines.

Keywords: HPLC method, gatifloxacin, fluorometholone, validation, ICH guidelines

INTRODUCTION:

Gatifloxacin sesquihydrate (GATI) **(Figure 1) [1]** chemically known as, 1-cyclopropyl-6-fluoro-8-methoxy- 7- (3-methylpiperazin-1-yl)- 4- oxo -1, 4- dihydroquinoline- 3-carboxylic acid sesquihydrate. This antibiotic belongs to the 4th generation of fluoroquinolones and works by inhibiting topoisomerase IV and DNA gyrase, two bacterial enzymes.

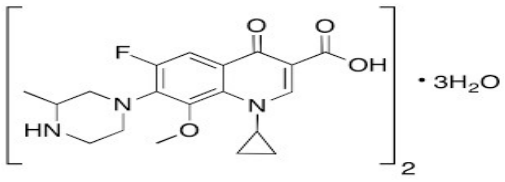

Figure 1: Gatifloxacin sesquihydrate

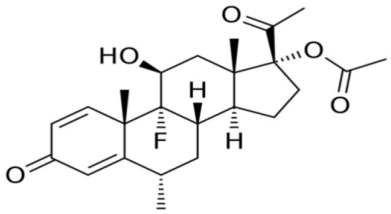

Figure 2: Fluorometholone acetate

Fluorometholone acetate (FLM) **(Figure 2) [2]** chemically named as 9- Fluoro-11ß, 17-dihydroxy-6-methylpregna-1,4-diene- 3,20 dione, 17 acetate16. It is a glucocorticoid, employed as eye drops, in the treatment of allergic and inflammatory conditions of the eye.

Extensive literature survey shows that analytical methods like spectrophotometric and HPLC are available for the estimation of these drugs individually or in combination with other drugs. But a very few methods are available for the simultaneous estimation of these drugs. Hence we tried to develop new and simple spectrophotometric and HPLC methods for the simultaneous estimation of these drugs. The developed methods were validated as per guidelines of ICH guidelines **[3]**.

MATERIALS AND METHODS:

Drug:

Gatifloxacin sesquihydrate (purity-97.7%) - Micro labs ltd.

Fluorometholone acetate (purity-98.5%) - Micro labs ltd.

Table 1: Reagents and chemicals used

S. No.	Chemical/Solvent	Grade	Make
1	Methanol & Acetonitrile	HPLC grade	Merck
2	Water	HPLC grade	Milli Q
3	Potassium dihydrogen phosphate & Hydrochloric acid	AR grade	Emplura
4	Sodium hydroxide	AR grade	Merck
5	Hydrogen peroxide & Ortho phosphoric acid	AR grade AR grade	Qualigens

UV Spectrophotometric method [4, 5]

Selection of solvent:

Mixture of methanol and water (70:30 v/v) was used as the solvent for dissolving GATI and FLM

Preparation of standard solutions:

Weigh accurately 10 mg of each API and transferred into 100 ml V.F and add diluent [MeOH: water (70:30)], then sonicated to dissolve the contents. Further dilute 10 ml of above stock solutions to 100ml with diluent separately to obtain 10 µg/ml concentrations of GATI and FLM separately.

Determination of ⋋max:

Both the standard solutions were scanned separately between 400nm to 200nm in 1 cm cell against blank. The individual UV absorbance spectrum of GATI is 290nm and FLM is 240nm. From the overlain spectrum the wavelength selected for estimation of drug was 274nm as isoabsorptive point was shown in **Figure 3**.

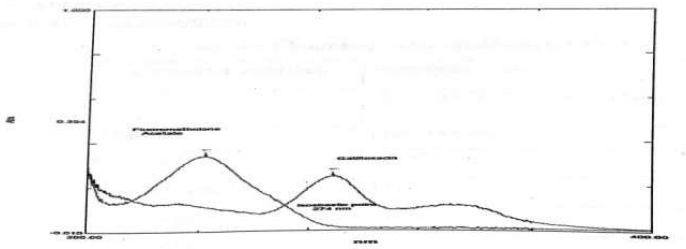

Figure 3: The overlain UV spectra of GATI and FLM

Method development by HPLC: [6, 7]

Preparation of standard stock solution:

Accurately weighed and transferred 32.2 mg of GATI (equivalent to 30 mg of gatifloxacin) and 10.0mg of FLM of both working standard into 100 ml of volumetric flask, added about 70 ml of diluent, sonicated to dissolve, dilute to the mark with the diluent and mix well. Further diluted 5.0 ml of above filtrate to 25 ml with diluent as stock solution 2.

Preparation of buffer: (Phosphate buffer pH 3) **[8]**

Mobile phase A: Phosphate buffer pH 3.0.

Mobile phase B:

Acetonitrile (ACN): Methanol (MeOH) (60:40).

Optimized chromatographic condition:

Table 2: Optimized chromatographic condition

Parameter/ conditions	Description / values
Column name	Hypersil Gold C18 column, 4.6 x 250 nm, 5µ
HPLC system	Waters alliance
Detector	PDA detector
Flow rate	1.0 ml/min
Injection volume	10µl
Wavelength	GATI: 290nm and FLM: 240nm
Column temperature	25℃
Run time	20 min
Mobile phase	Buffer: [ACN/ MeOH (60:40)]
Program	Gradient
Diluent	MeOH: water (70:30)

HPLC method validation for determination of GATI and FLM according to ICH guidelines [3]:

Validation is a process of "Establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result (or) product meeting its pre-determined specifications and quality attributes" The following parameters were considered for the analytical method validation for determination of GATI and FLM . They are similarity factor, precision, linearity, range, accuracy, specificity/selectivity, ruggedness, and robustness.

Similarity factor:

According to standard procedure two standard Solutions were prepared, and result obtained by using HPLC. Shown in **Figure 4 & 5**

Calculation:

$$\textbf{Similarity factor} = \frac{\textbf{Weight of Standard 1}}{\textbf{Weight of Standard 2}} \textbf{X} \frac{\textbf{Area of Standard 2}}{\textit{Area of Standard 1}}$$

Limit: (0.98-1.02)

System suitability:

System suitability test was used to verify, whether the resolution & reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from 5 replicate injections of standard drug solution.

Linearity study:

Linearity was performed by diluting std. stock solution. From stock solution aliquots of both std. drugs is taken and the final concentration of GATI in the range of 15 to 90 µg/mL (25-150%) and FLM in the range of 5 to 30 µg/mL (25- 150%). 10 µl of each sample injected in duplicate for each concentration level and calibration curve was constructed by the peak area vs concentration. Shown in **Figure 6 & 7 & Table 3**.

Precision:

1. System precision:

The system precision is checked by using std. GATI and FLM to ensure that the analytical system is precise. The retention time and area of ten determinations were measured and RSD was calculated. 10µl of mixed standard solution was injected ten times separately and their system suitability parameters were recorded.

2. Method precision:

In method precision, a homogenous sample of a single batch should be analyzed six times. This indicates whether a method is giving consistent results for a single batch. Shown in **Figure 8 & 9 & Table 4**.

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy of

proposed method was ascertained on the basis of recovery study performed by standard addition method. Accuracy was performed in three different levels for GATI and FLM. Analyses of samples were done in triplicate for each level. From the results, % recovery was calculated. Shown in **Figure 10 & 11 & Table 5**.

Calculation:-

$$\text{µg/mL recovered} = \frac{\text{Area of Sample}}{\text{Area of Std}} \text{ x } \frac{\text{Weight of Std Potency}}{\text{Std Dilution}} \text{x } \frac{\text{Potency}}{100} \text{x} \frac{Factor\ 1}{Factor\ 2} \text{x} 1000$$

$$\text{\% Recovery} = \frac{\text{Area of Sample}}{\mu g/mL\ added}$$

Range:

Range to be deduced using precision experiment, linearity, and recovery data.

Specificity and selectivity:

The analytes must be well-resolved from any extraneous components and free from interference. Whereas selectivity is the process to detect the analyte qualitatively in the presence of components that may be expected to be present in the sample matrix, specificity is the process to detect the analyte quantitatively in the presence of those components. Shown in **Figure 12 & 13 & Table 6**

Ruggedness:

The study of ruggedness was carried out under following different conditions: Shown in **Table 7**.

Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Shown in **Table 8**.

RESULTS AND DISCUSSION:

Similarity factor:

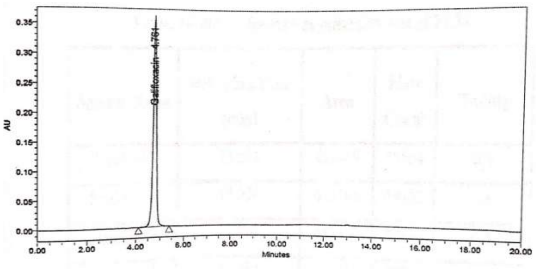

Figure 4: HPLC Chromatogram of similarity factor of GATI Std at 290 nm

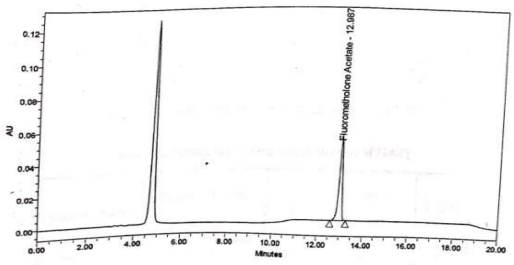

Figure 5: HPLC Chromatogram of similarity factor of FLM Std at 240 nm

Linearity study:

Table 3: Result and statistical data of linearity of GATI & FLM

Si. no	Linearity level (%)	Vol. of stock taken	diluted	Conc. (ppm) of GATI	Avg. area of GATI	Conc. (ppm) of FLM	Avg. area of FLM
1	25	1.25	25	14.6550	860331	4.9350	116735
2	50	2.5	25	29.3100	1660723	9.8500	224145
3	75	3.75	25	43.9650	2576532	14.7750	344336
4	100	5	25	58.6200	33352222	19.7000	449680
5	125	6.25	25	73.2750	4132459	24.6350	551005
6	150	7.5	25	87.9300	4949657	29.5500	662305
		Correlation coefficient (R^2)			0.9995		0.9995
		Slope (m)			817729		108966
		Intercept (y)			57101.2000		9983.6

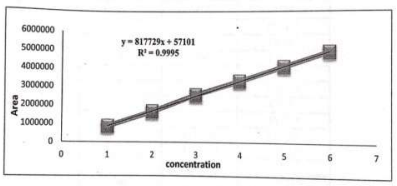

Figure 6: HPLC Linearity of GATI at 290nm

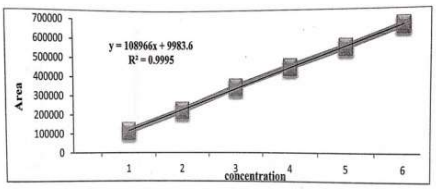

Figure 7: HPLC Linearity of FLM at 240nm

Precision:

Method precision:

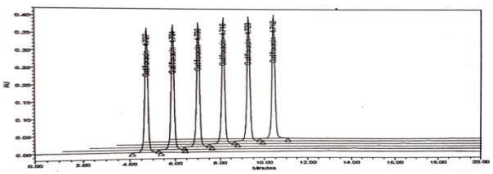

Figure 8: Overlain HPLC Chromatogram for Method precision of GATI at 290nm

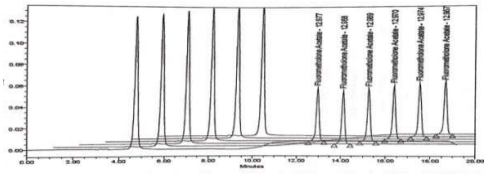

Figure 9: Overlain Chromatogram Precision for Method Precision of FLM at 240nm

Table 4: Results of Method Precision of GATI and FLM

Injection no.	GATI	FLM
	Area	Area
1	3301406	450103
2	3300635	449919
3	3301562	450997
4	3302986	453816
5	3300897	449712
6	3300022	449198
Mean	3301251	450624.2
S.D	1014.513	1671.028
% RSD	0.03	0.37

Accuracy:

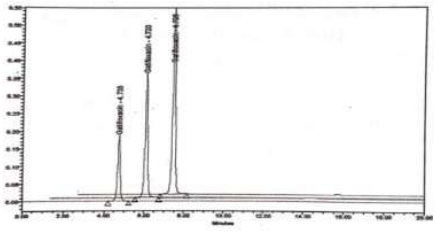

Figure 10: Overlain HPLC Chromatograms for Method Accuracy of GATI at 290nm

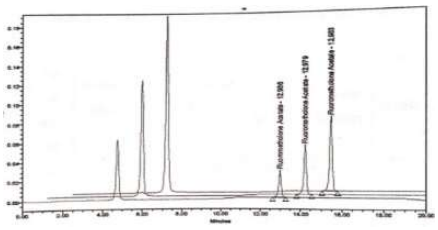

Figure 11: Overlain Chromatograms for Method Accuracy of FLM at 240nm

Table 5: Result and Statistical data of Accuracy of GATI at 290nm & FLM at 240nm

Conc. Level %	Vol. of stock sol	Amt spiked (µg)		Avg. area		Amt recovered		% recovery (µg)		% RSD	
		GATI	FLM	GATI	FLM	GATI	FLM	GATI	FLM	GATI	FLM
50	2.5	31.4887	9.879	1690004	223347	31.5825	9.7863	100.3	99.1	0.2	1.1
50	2.5	31.4887	9.879	1694437	228163	31.6653	9.9973	100.6	101.2		
50	2.5	31.4887	9.879	1693642	227590	31.6505	9.9722	100.5	100.9		
100	5.0	62.9774	19.75	3362619	446874	62.8400	19.5804	99.8	99.1	0.2	0.5
100	5.0	62.9774	19.75	3377755	446988	63.1229	19.5854	100.2	99.1		
100	5.0	62.9774	19.75	3361828	451095	62.8253	19.7654	99.8	100.0		
150	7.5	94.4661	29.63	5033637	669019	94.0677	29.3140	99.6	98.9	0.9	0.1
150	7.5	94.4661	29.63	4964888	669198	92.7830	29.3219	98.2	98.9		
150	7.5	94.4661	29.63	4956047	669390	92.6178	29.3303	98.0	99.0		

Specificity and selectivity:

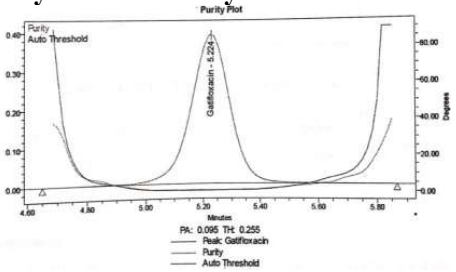

Figure 12: GATI Purity Plot at 290nm

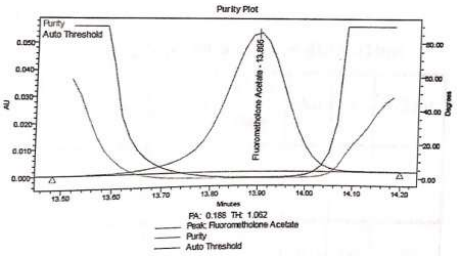

Figure 13: FLM Purity Plot at 240nm

Table 6: Result of Peak Purity of GATI and FLM

Peak	Purity angle	Purity threshold
GATI	0.095	0.225
FLM	0.188	1.062

Ruggedness:

Table 7: Result of Ruggedness Study of GATI at 290nm & FLM at 240nm

Test parameter	condition	%label claim of GATI	Results			%label claim of FLM	Results		
			Mean	S.D	% RSD		Mean	S.D	% RSD
Intra day	0 hrs	100.3	100.15	0.21	0.209	100.0	100.95	0.07	0.06
	4 hrs	100.0				100.9			
Inter day	Day 1	100.3	100.15	0.91	0.908	101.0	99.95	1.48	1.48
	Day 2	99.0				98.9			
Different analyst	Analyst 1	101	100.75	0.35	0.347	100.71	99.95	1.06	1.06
	Analyst 2	100.5				99.2			
Different column	Column 1	100.0	99.85	0.21	0.210	102	101.5	0.70	0.69
	Column 2	99.7				101			
Different system	System 1	100.7	99.85	1.20	1.20	102.0	101.95	0.70	0.69
	System 2	99.7				101.0			

Robustness:

Effect of variation in temperature of column and flow rate

Table 8: Result of System suitability parameters

S. No.	System suitability parameter		Observations for temperature			Observations for flow rate		Limits
			As such	(20°C) -5°C	(20°C)+5°C	0.9 ml-0.1 ml	1.1 ml+0.1 ml	
1	% RSD	GATI	0.5	0.4	0.6	0.2	0.5	NMT 2.0
		FLM	0.5	0.4	0.6	0.3	0.5	
2	Theoretical plates	GATI	7414	7222	7145	7992	6564	NLT 2000
		FLM	54911	47426	47329	51723	50582	
3	Tailing factor	GATI	1.0	0.95	0.95	1.0	1.0	NMT 2.0
		FLM	0.8	0.85	0.84	0.8	0.8	
4	Retention time	GATI	5.200	5.865	5.420	6.258	4.898	
		FLM	13.866	15.772	13.757	15.360	13.334	

Table 9: Results summary of Method Validation for GATI and FLM

S. No.	Parameter	Requirement	Results		Acceptance criteria
			GATI	FLM	
1	System suitability	RT	4.776	13.024	
		Plate count	6191	48564	NLT 2000
		Tailing factor	0.8	0.9	NMT 2
2	Accuracy	% recovery	99.7%	99.6%	98-102%
3	Precision	% RSD	0.2	0.5	NMT 2%
4	Specificity	No interference	Pass	Pass	No interference
5	linearity	Correlation coefficient	0.999	0.999	NLT 0.999
6	Range	Concentration (ppm)	15-90	5-30	Nil
7	Robustness	% RSD	0.4	0.3	NMT 2%
8	Ruggedness	% RSD	0.5	0.4	NMT 2%

CONCLUSION:

From this studies, it was concluded that HPLC technique can be successfully used for the estimation of GATI and FLM in their combined dosage ophthalmic formulations. The developed method was validated in terms of accuracy, precision, linearity, specificity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The method shows good reproducibility and it is accurate, precise, specific and sensitive. No interference of additives, matrix etc. is encountered in this method. The method was found to be sensitive, reliable, reproducible, rapid and economic also.

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