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METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF MOXIFLOXACIN HYDROCHLORIDE AND LOTEPREDNOL ETABONATE OPTHALMIC SUSPENSION BY RP-HPLC.

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Abstract: For the estimation of Moxifloxacin Hydrochloride and Loteprednol Etabonate in pharmaceutical tablet dosage forms an accurate, simple, rapid, efficient, highly sensitive, precise Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed and validated. Hypersil Gold column (250mm x 4.6mm; 5µ) was used in gradient mode to develop the RP-HPLC method. The mobile phase A (Buffer) contained potassium dihydrogen ortho phosphate pH adjusted to 3.0 with ortho phosphoric acid. The mobile phase B (Organic phase) contained a 60:40 v/v ratio of acetonitrile and methanol. The flow rate was 1.0ml/min, and the effluent was measured at 274nm UV wavelength using a PharmaSpec UV-1700 UV-Vis spectrophotometer. The retention time of Moxifloxacin was 9.541 and Loteprednol was 14.210 minutes. According to ICH guidelines, the method was validated with respect to system suitability, specificity, precision, accuracy, linearity and range, robustness and ruggedness. The linearity with range of Moxifloxacin Hydrochloride was found to be 0.03015 mg/ml to 0.09045 mg/ml, with a correlation coefficient of 0.9999. The linearity with range of Loteprednol Etabonate was found to be 0.05015 mg/ml to 0.15045 mg/ml, with a correlation coefficient of 0.9999. The developed method was successfully used to quantify the bulk and active pharmaceutical ingredient content of tablet dosage form.

Keywords: **Moxifloxacin Hydrochloride, Loteprednol Etabonate, UV-Visible Spectrophotometer, Validation Methods.**

INTRODUCTION

Moxifloxacin Hydrochloride chemically designated as (1'S,6'S)-1-Cyclopropyl-7-(2,8-diazabicyclo[4.3.0]non-8-yl)-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid hydrochloride is a category of fluoroquinoline and antibactetial with molecular formula $C_{21}H_{24}FN_3O_4$.HCl [1,2]. It is a yellow crystalline powder which is sparingly soluble in water, slightly soluble in ethanol and insoluble in acetone [3]. Moxifloxacin is a broad spectrum antibiotic that is active against both gram positive and gram negative bacteria. It is sold as an ophthalmic solution as the brand name of Vigamox for the treatment of conjunctivitis (pink eye). It is also used for Respiratory tract infection, Skin infection, Intra-abdominal infection, Endocrarditis, Anthrax, Meningitis, and Tuberculosis [4].

Loteprednol Etabonate chemically designated as [17-(ethyl carbonate) chloromethyl 11β, 17-dihydroxy-3-oxoandrosta-1,4-diene-17β-carboxylate] is a category of anti-inflammatory and anti-allergic agents with molecular formula $C_{24}H_{31}ClO_7$. It is a white crystalline powder which is freely soluble in chloroform, sparingly soluble in acetone, slightly soluble in methanol and insoluble in water [5]. It is a steroids and steroid derivatives, which may induce phospholipase A2 inhibitory proteins: these proteins may inhibit the release of arachidonic acid and thus control biosynthesis of potent mediator of inflammation. It is used in

ophthalmic solution for the treatment of steroid responsive inflammatory conditions of eye such as allergic conjunctivitis, uveitis, acne rosacea, superficial punctate keratitis, iritis, cyclitis. As a nasal spray used for the treatment of seasonal allergic rhinitis [6].

According to the literature survey, there are no establish methods available for the determination of Moxifloxacin Hydrochloride and Loteprednol Etabonate in combination, the present work is an attempt to estimate the combination by different method such as RP-HPLC and UV-Vis Spectrophotometry [7]. There is a need to develop and validate a new simple, sensitive, accurate and economical analytical method for analysis of Moxifloxacin Hydrochloride and Loteprednol Etabonate ophthalmic suspension by RP-HPLC. Validate the purposed RP-HPLC method in accordance with USP and ICH guideline [8].

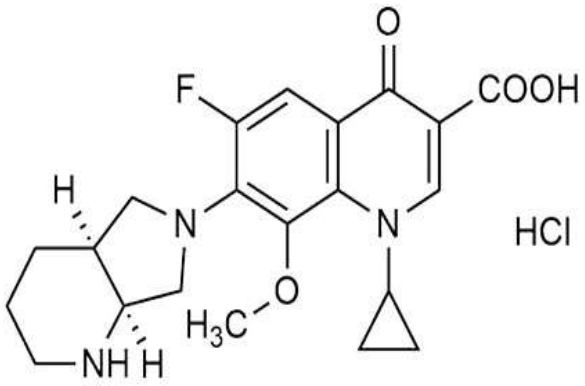

Figure 1**:** Chemical structure of Moxifloxacin Hydrochloride

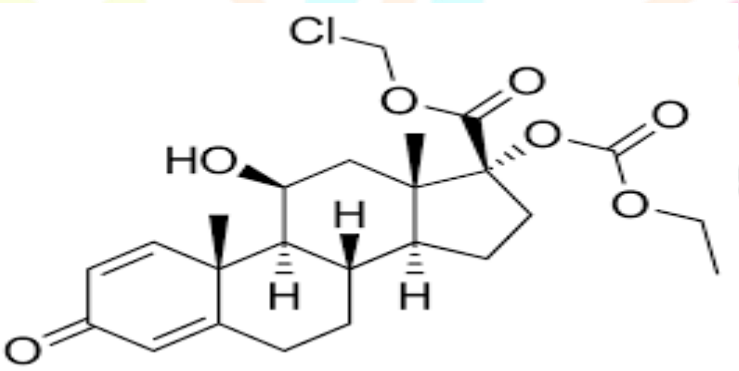

Figure 2**:** Chemical structure of Loteprednol Etabonate

MATERIALS AND METHODS

MATERIALS

Shimadzu module HPLC was used which has LC-solution software and UV detector, UV Visible spectrophotometry of PharmaSpec UV-1700 model with UV-probe software was used. Chemical reagents like Potassium dihydrogen ortho phosphate, Acetonitrile, Methanol from Merck; Orthophosporic acid from Spectrochem, Water from Mili Q were used.

WAVELENGTH SELECTION

Weighed accurately 50mg of Moxifloxacin API in a 50ml volumetric flask then dissolved in diluent (methanol and water in 68:32 ratio) with the help of sonication and make up with diluent up to the mark (1000ppm). 5ml of this solution was diluted in a 50ml volumetric flask with diluent (100ppm). 4ml of this dilute solution was further diluted with the diluent (8ppm). This 8ppm solution was used for scanning purpose.

Weighed accurately 50mg of Loteprednol API in a 50ml volumetric flask then dissolved in diluent (methanol and water in 68:32 ratio) with the help of sonication and make up with diluent up to the mark (1000ppm). 5ml of this solution was diluted in a 50ml volumetric flask with diluent (100ppm). 6ml of this dilute solution was further diluted with the diluent (12ppm). This 12ppm solution was used for scanning purpose.

The absorbance of solutions was scanned in the UV range of 200 to 400nm & it was detected at 274nm.

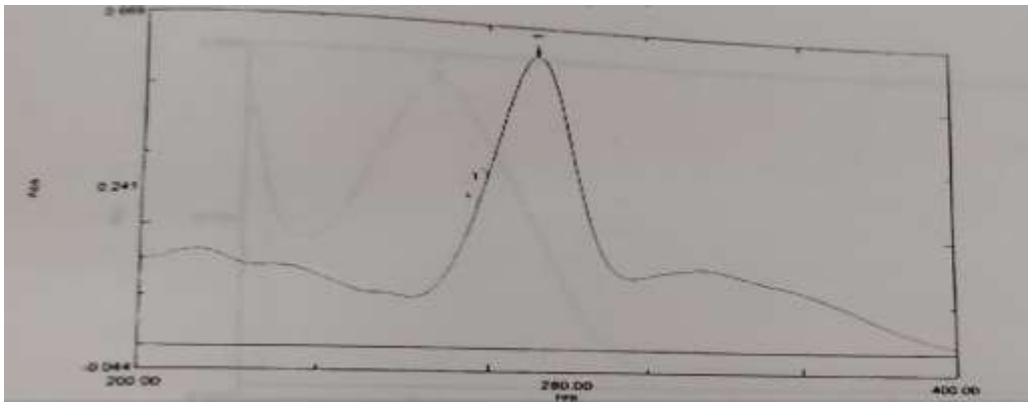

Figure 3**:** Typical UV Spectrum of Moxifloxacin Hydrochloride

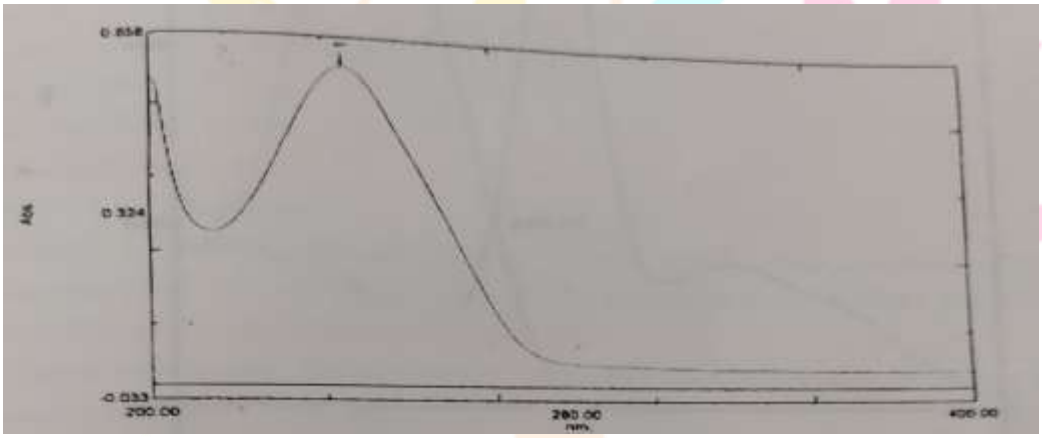

Figure 4**:** Typical UV Spectrum of Loteprednol Etabonate

METHOD DEVELOPMENT BY RP-HPLC

Table 1: Chromatographic condition

Column	Hypersil Gold
Column Dimension	250mm x 4.6mm; 5µ
Detector	PDA Detector
Wavelength	274 nm
Flow rate	1.0 ml/min
Injection Volume	50 µl
Column Oven Temp	35°C
Sample Cooler Temp	10°C
Run time	20 min

SELECTION OF MOBILE PHASE

Mobile Phase A (Buffer): About 3.2gm of Potassium Dihydrogen Ortho Phosphate dissolved in 1000ml of water and pH adjusted to 3.0 with ortho phosphoric acid; filtered through 0.45µ nylon membrane filter.

Mobile Phase B (Organic Phase): Mixed Acetonitrile and Methanol in 60:40 v/v ratio and sonicated to degas.

Diluent: Mixed methanol and water in the ratio of 68:32 v/v; sonicated to degas.

PREPARATION OF STANDARD SOLUTION

Moxifloxacin Stock: Accurately weighed 60.0mg of Moxifloxacin Hydrochloride and 100.0mg of Loteprednol Etabonate were transferred to 100ml volumetric flask. To this 68ml of methanol was added and sonicated to dissolve then make up to mark with water.

Further diluted 5ml in 50ml volumetric flask and diluted up to the mark with diluent.

PREPARATION OF SAMPLE

Weigh accurately equivalent to 3mg of Moxifloxacin Hydrochloride and 5mg of Loteprednol Etabonate into 50ml volumetric flask, make up volume with diluent and mix well.

Table 2: Gradient Program

Time (min)	Mobile Phase A	Mobile Phase B
0	73	27
5	73	27
10	27	73
15	27	73
18	73	27
20	73	27

Observation: RT of Moxifloxacin – 9.541

RT of loteprednol – 14.210

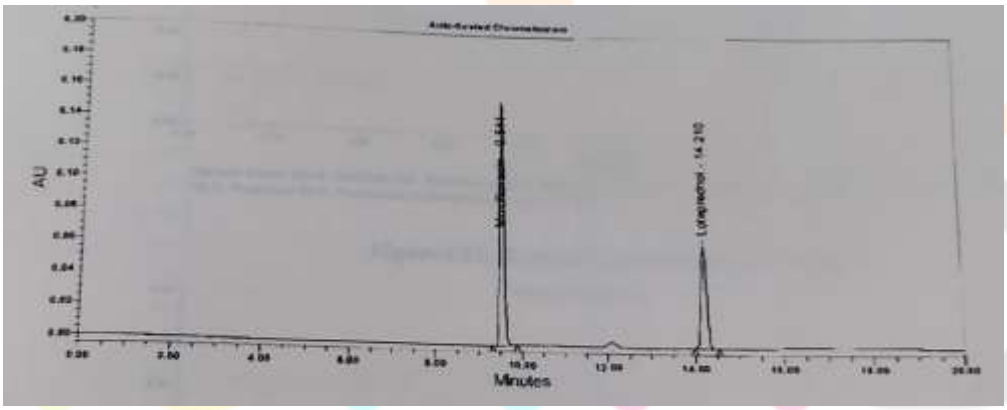

Figure 5: Typical Chromatogram for Developed method

Conclusion: Both peaks pass the system suitability parameters
Peak purity is also passed

RESULTS AND DISCUSSION

RESULTS OF HPLC METHOD

The following parameters were considered for analytical method validation of Moxifloxacin Hydrochloride and Loteprednol Etabonate [9,10].

- System Suitability
- Linearity and range
- Precision
- Accuracy
- Specificity
- Robustness and ruggedness

SYSTEM SUITABILITY

To verify the analytical system is working properly and can give accurate also precise results, the system suitability parameters are to be set. Injected the standard solution and recorded the chromatogram.

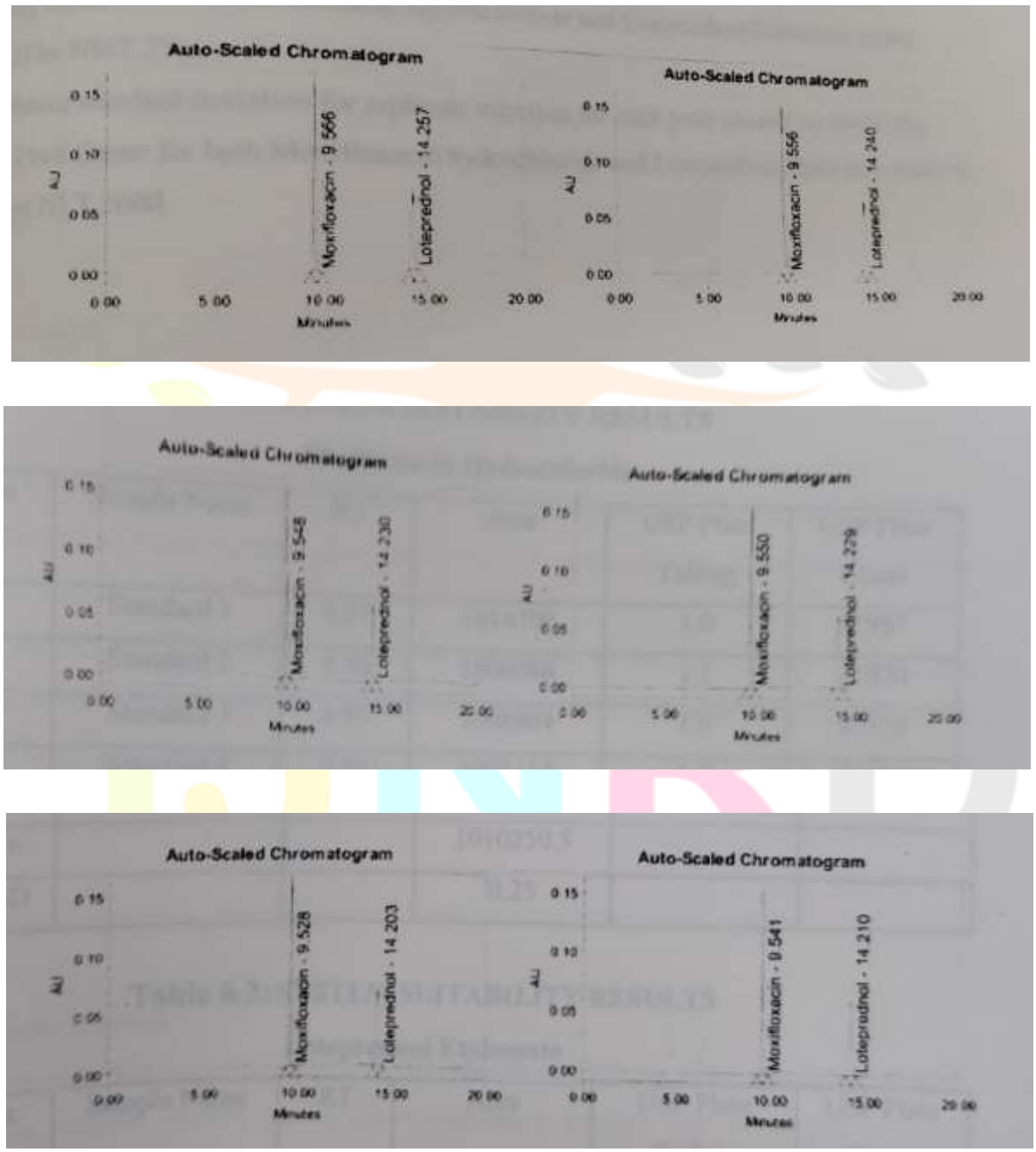

Figure 6**:** Typical Chromatogram of standards (system suitability)

Acceptance Criteria:

- Tailing factor for both Moxifloxacin Hydrochloride and Loteprednol Etabonate peaks should be NMT 2%.
- %Relative standard deviation for replicate injection for each peak should be NMT 2%.
- USP plate count for both Moxifloxacin Hydrochloride and Loteprednol Etabonate peak is always NLT 2000.

Table 3: Moxifloxacin Hydrochloride system suitability results

S. No.	Sample Name	RT	Area	USP Plate Tailing	USP Plate Count
1	Standard 1	9.57	1014706	1.0	47957
2	Standard 2	9.56	1008088	1.1	47020
3	Standard 3	9.55	1008904	1.0	47679
4	Standard 4	9.56	1008167	1.0	47619
5	Standard 5	9.53	1011223	1.0	47296
6	Standard 6	9.54	1010415	1.1	46836
Mean			1010250.5		
%RSD			0.25		

Table 4: Loteprednol Etabonate system suitability results

S. No.	Sample Name	RT	Area	USP Plate Tailing	USP Plate Count
1	Standard 1	14.26	566055	1	59774
2	Standard 2	14.24	563939	1	59971
3	Standard 3	14.23	562674	1	60806
4	Standard 4	14.23	562987	1	61135
5	Standard 5	14.20	565689	1	60726
6	Standard 6	14.21	564263	1	60745
Mean			564267.9		
%RSD			0.24		

Conclusion: It is observed from above tabulation, that the method complies with system suitability parameters. Hence, the parameter meets the requirement of method validation.

LINEARITY AND RANGE

The linearity of an analytical method is its ability to elicit test results that are directly or mathematically proportional to the concentration of analyte in samples within a given range. Individually samples equivalent to 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150% of the stated amount of sample were weighed individually and the assay was carried out [11,12].

A graph of concentration versus area was plotted for both Moxifloxacin Hydrochloride and Loteprednol Etabonate peaks. The regression line obtained was linear. From data obtained, co-relation coefficient, slope, y-intercept were calculated. Ideally co-relation coefficient should be around 1.

Standard stock solution was prepared by accurately weighed 60.0mg of Moxifloxacin Hydrochloride and 100mg of Loteprednol Etabonate. Then, transferred the above mentioned weights to two different 100ml volumetric flask. To this add 68ml of methanol and sonicate to dissolve and make up to mark with water.

Table 5: Linearity and range of Moxifloxacin Hydrochloride

Linearity Level (%)	Vol. of stock taken	Diluted	Concentration (ppm)	Avg. area
50	2.5	50	30.15	492471
60	3.0	50	36.18	600339
70	3.5	50	42.21	692260
80	4.0	50	48.24	794571
90	4.5	50	54.27	893329
100	5.0	50	60.30	1002118
120	6.0	50	72.36	1205591
130	6.5	50	78.39	1304352
140	7.0	50	54.42	1407880
150	7.5	50	90.45	1494302
	Co-relation Coefficient			0.9999
	Slope (m)			190238.7801
	Intercept (y)			-10644.8646

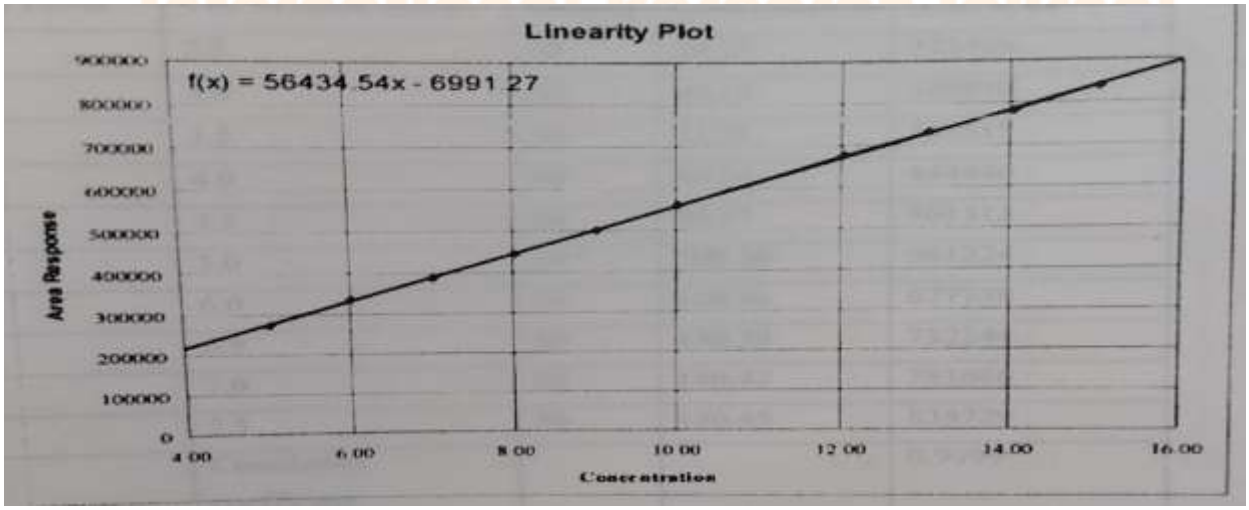

Figure 7**:** Calibration curve of Moxifloxacin Hydrochloride

Conclusion: The method was found to be linear with the range of 0.03015mg/ml to 0.09045mg/ml and the co-relation coefficient of the plot for Moxifloxacin Hydrochloride was 0.9999.

Table 6: Linearity and range of Loteprednol Etabonate

Linearity Level (%)	Vol. of stock taken	Diluted	Concentration (ppm)	Avg. area
50	2.5	50	50.15	273303
60	3.0	50	60.18	335650
70	3.5	50	72.21	387515
80	4.0	50	80.24	444446
90	4.5	50	90.27	501313
100	5.0	50	100.30	561224
120	6.0	50	120.36	677758
130	6.5	50	130.39	732244
140	7.0	50	140.42	781686
150	7.5	50	150.45	838729
	Co-relation Coefficient			0.9999
	Slope (m)			56434.5421
	Intercept (y)			-6991.2727

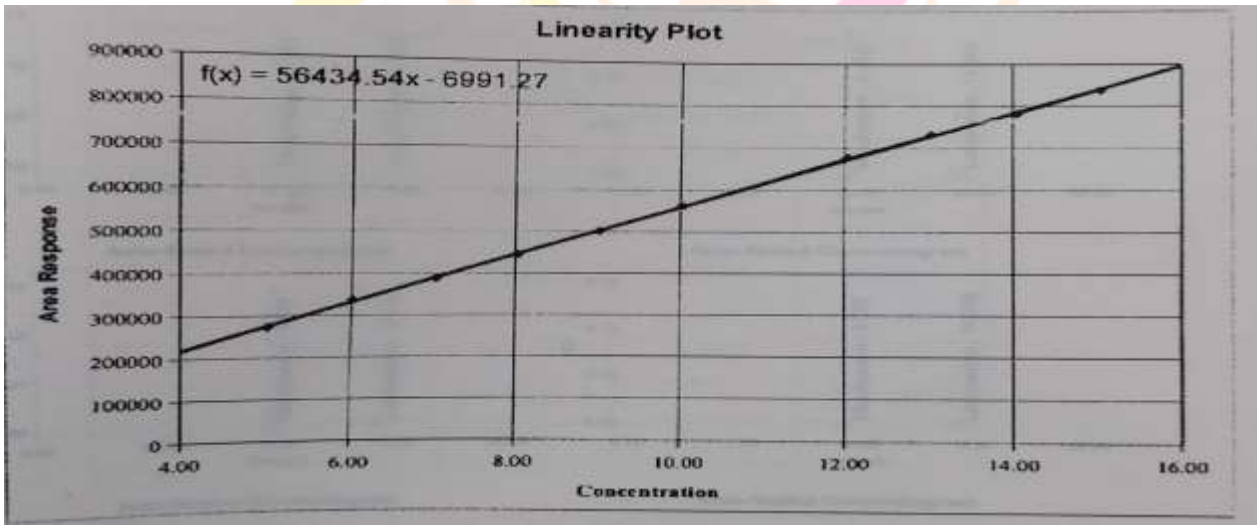

Figure 8**:** Calibration curve of Loteprednol Etabonate

Conclusion: The method was found to be linear with the range of 0.05015mg/ml to 0.15045mg/ml and the co-relation coefficient of the plot for Loteprednol Etabonate was 0.9999.

PRECISION

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. The precision is usually expressed the standard deviation or relative standard deviation of series of measurement.

In method precision, a homogenous sample of single batch should be analyzed six times. This indicates whether a method is giving consistent results for a single batch. The method precision was performed on Moxifloxacin Hydrochloride and Loteprednol Etabonate formulation [13].

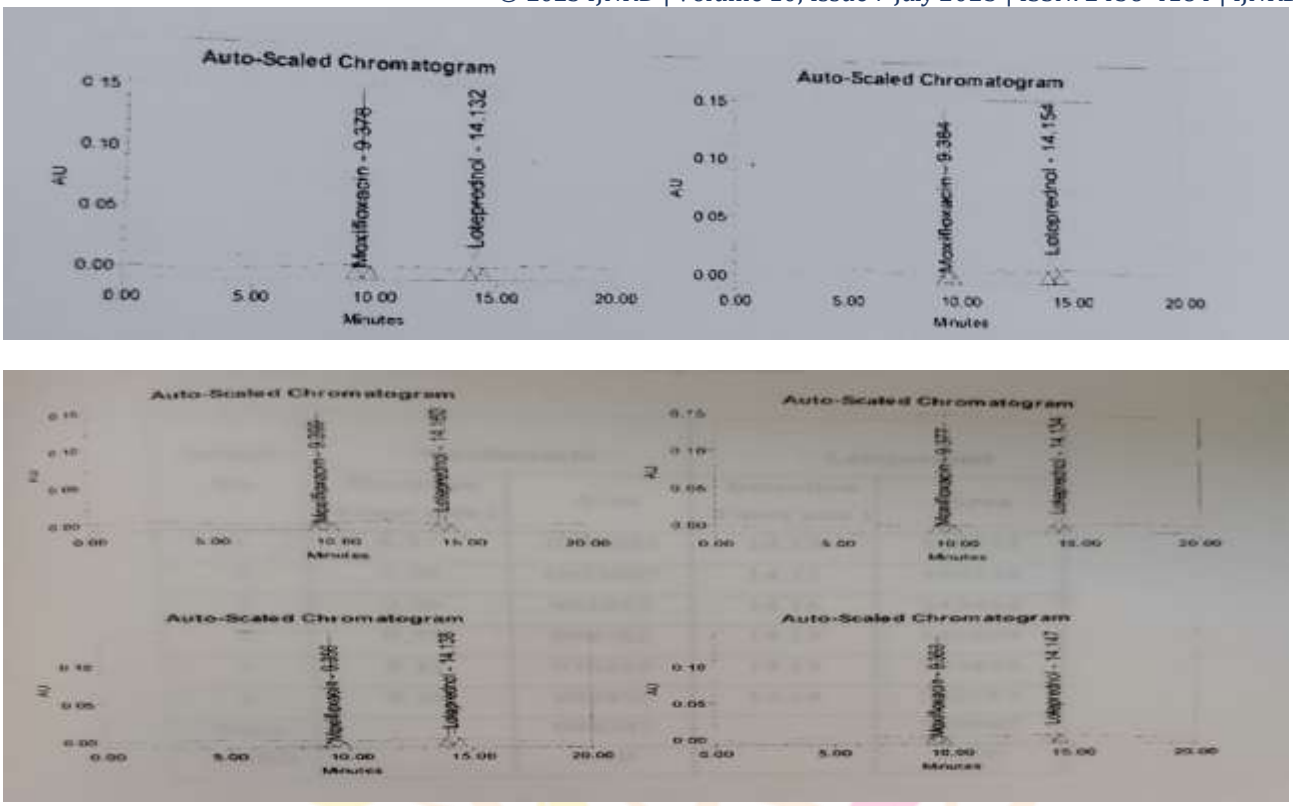

Figure 9**:** Typical Chromatogram for method precision

Table 7: Results of method precision

Sample No.	Moxifloxacin		Loteprednol	
	Retention Time (min)	Area	Retention Time (min)	Area
1	9.37	1003691	14.13	555551
2	9.38	1003007	14.15	556754
3	9.39	985857	14.16	543454
4	9.37	998765	14.13	545898
5	9.35	976265	14.13	543438
6	9.35	998495	14.14	552183
Mean		994347		549547
% RSD		1.10		1.10

Conclusion: From the above result, it was concluded that the method is precise.

READINGS OF ACCURACY

The accuracy of an analytical method is the closeness of test results obtain by the method to the true value. Performed accuracy in three different levels for Moxifloxacin Hydrochloride and Loteprednol Etabonate. Spiked known quantity of Moxifloxacin Hydrochloride and Loteprednol Etabonate standard at 50%, 100% and 150% level into the placebo. Analyze these samples in triplicate for each level.

Table 8: Results for recovery of Moxifloxacin Hydrochloride

Sr. No.	Level in %	Response Area	% Recovery	Average Recovery	% RSD
1	50	556338	101.0	101.0	0.0
2	50	556433	101.0		
3	50	556515	101.0		
4	100	1083775	98.3	98.3	0.0
5	100	1083795	98.3		
6	100	1083817	98.3		
7	150	1630173	98.6	98.6	0.0
8	150	1630168	98.6		
9	150	1630165	98.6		
Overall Recovery				99.3	1.3

Table 9: Results for recovery of Loteprednol Etabonate

Sr. No.	Level in %	Response Area	% Recovery	Average Recovery	% RSD
1	50	276840	98.1	98.3	0.2
2	50	277650	98.4		
3	50	279816	99.2		
4	100	562470	99.7	99.8	0.1
5	100	562886	99.8		
6	100	562453	99.7		
7	150	830002	98.1	98.2	0.1
8	150	830832	98.2		
9	150	798768	94.4		
Overall Recovery				98.4	1.7

Conclusion: From the above result, it was concluded that the recovery is well within the limit. Hence, the method is accurate.

SPECIFICITY

The peak purity test using diode array detector to show that the analyte chromatographic peak is not attributable to more than one component [14].

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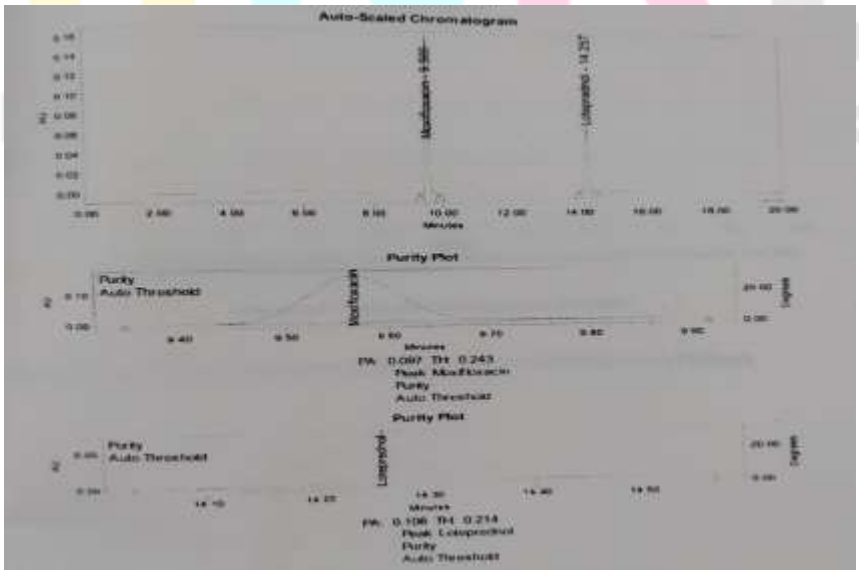

Table 10: Peak Purity

Peak	Purity Angle	Purity Threshold
Moxifloxacin	0.115	0.249
Loteprednol	0.078	0.214

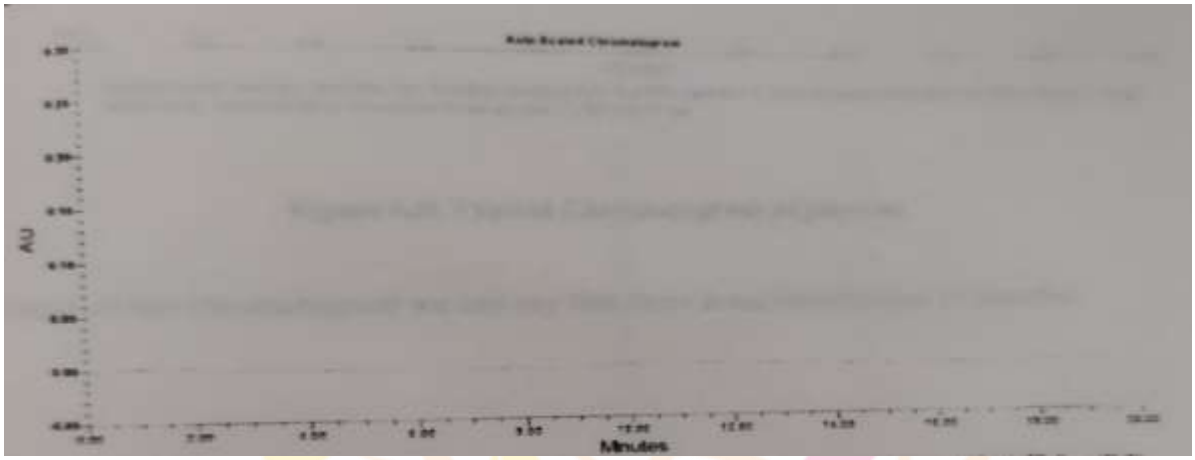

Figure 10**:** Typical Chromatogram of Blank

Conclusion: On the basis of this chromatogram, there is no interference of diluent.

ROBUSTNESS

The robustness of an analytical method is 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 [15].

Robustness was done by changing the column temperature (±5°C), flow rate (±10%).

Table 11: Standard area of Moxifloxacin Hydrochloride and Loteprednol Etabonate OS for robustness

Name	Area 1	Area 2	Area 3	Area 4	Area 5	% RSD
Moxifloxacin Hydrochloride	1014705	1008085	1008900	1008161	1010410	0.3
Loteprednol Etabonate	566033	563937	562675	562984	564262	0.2

Conclusion: From the above result, it can be concluded that the recovery is well within the limit. Hence, the method is robust.

RUGGEDNESS

Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and analyst to analyst [16].

Table 12: Ruggedness results of different analyst

S. No.	Standard Moxifloxacin Area	Test Moxifloxacin Area	Standard Loteprednol Area	Test Loteprednol Area
Analyst – 1	1099991	1096467	559982	558257
Analyst – 2	1099911	1099265	558998	557438
Average	1099951	1097866	559490	557848
Assay (in %)	96.5	96.1	99.5	99.2

Table 13: Ruggedness results at different instrument

S. No.	Standard Moxifloxacin Area	Test Moxifloxacin Area	Standard Loteprednol Area	Test Loteprednol Area
Instrument – 1	1093691	1096467	559551	558257
Instrument – 2	1093007	1099265	559754	557438
Average	1093349	1097866	559653	557848
Assay (in %)	97.8	96.1	99.5	99.2

Table 14: Ruggedness results at different column

S. No.	Standard Moxifloxacin Area	Test Moxifloxacin Area	Standard Loteprednol Area	Test Loteprednol Area
Column – 1	1089093	1087904	552987	552674
Column – 2	1089486	1099010	553019	552614
Average	1089289	1093457	553003	552644
Assay (in %)	97.4	95.7	98.3	98.2

Conclusion: From the above result, it can be concluded that the recovery is well within the limit. Hence, the method is rugged.

ESTIMATION OF WATER CONTENT BY KF (KARL-FISCHER)

Moxifloxacin Hydrochloride - Take 200mg Moxifloxacin Hydrochloride and measure the water content.

Result – 4.06%

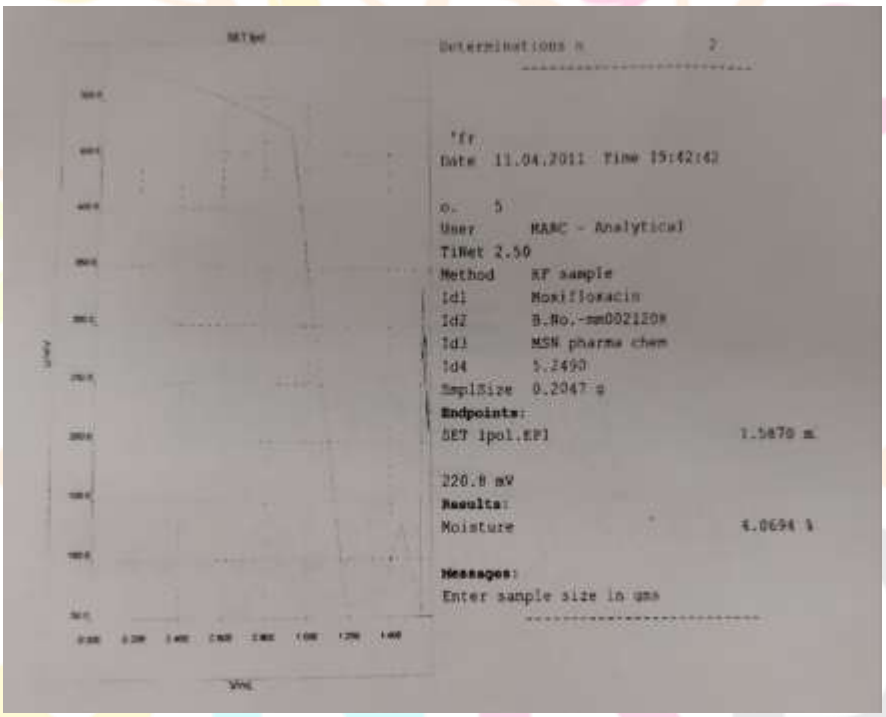

Figure 11: Water content of Moxifloxacin Hydrochloride

Loteprednol Etabonate - Take 200mg Loteprednol Etabonate and estimate the water content by KF Autotitrator with Tinet 2.5 software.

Result – 0.22%

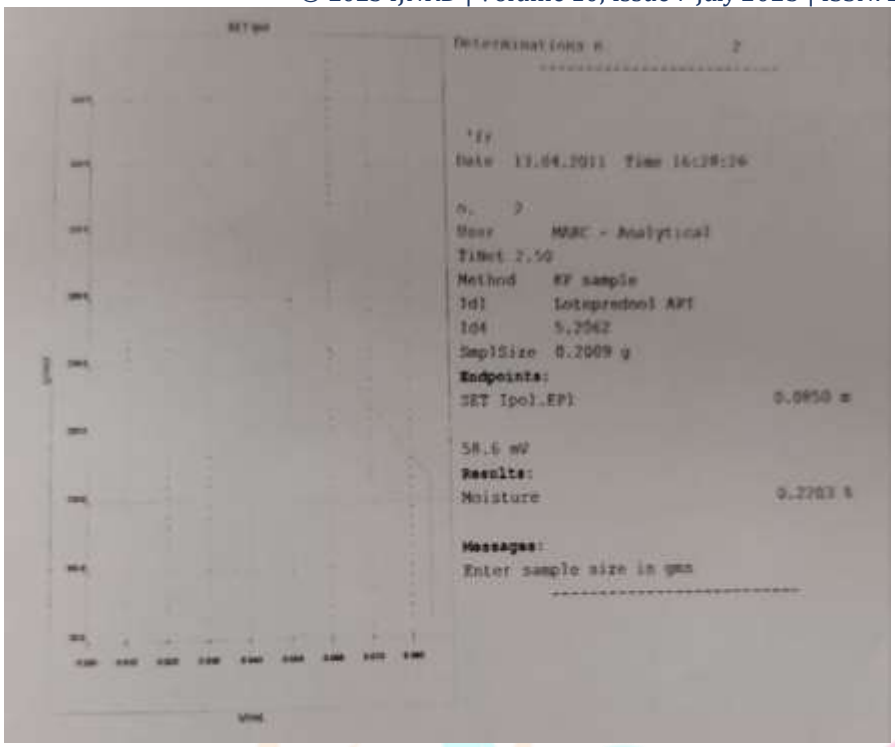

Figure 12: Water content of Loteprednol Etabonate

DISCUSSION

The literature survey revealed the combination of Moxifloxacin Hydrochloride and Loteprednol Etabonate. Many methods are available for the estimation of Moxifloxacin Hydrochloride and Loteprednol Etabonate. In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Mobile Phase and flow rate selection was based on peak parameters, run time, resolution. The mobile phase contained a 60:40 v/v ratio of acetonitrile and methanol. The flow rate was 1.0ml/min, and the effluent was measured at 274nm UV wavelength. The average retention time to be 9.541 min for Moxifloxacin and 14.210 min for Loteprednol. According to USP and IP, system suitability tests were an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. The calibration was linear in concentration range of 0.03mg/ml to 0.09mg/ml, with correlation coefficient 0.9999. The low values of % RSD indicate the method was precise and accurate. The mean recoveries were found in the range of 98-102%. Accuracy of the developed method for Moxifloxacin Hydrochloride and Loteprednol Etabonate are within the acceptance criteria of 98% to 102%. Robustness and ruggedness were found within the limit. Water content of Moxifloxacin Hydrochloride and Loteprednol Etabonate were determined by using KF Autotitrator and were found to be within the limit.

CONCLUSION

Since all the acceptance criteria of the parameters selected for validation are satisfied, the method stands validated. The method is simple, precise, accurate and rapid technique for determination of Moxifloxacin Hydrochloride and Loteprednol Etabonate ophthalmic suspension. The analytical method was developed based on HPLC. As the method have been validated according to USP and ICH guidelines.

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