Acta Scientific Pharmaceutical Sciences (ISSN: 2581-5423)

Volume 2 Issue 11 November 2018

Research Article

Development and Validation of Stability Indicating HPLC Method for the Determination of Fluoromethalone in Eye drops Formulations

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Received: September 20, 2018; **Published:** October 04, 2018

Abstract

Objective: A simple, precise and accurate stability indicating method was developed and validated for the analysis of Fluoromethalone in eye drop formulations.

Materials and Methods: Waters 2695 separations module and Waters 2695 PDA detector with Empower pro-software was operated on a Pentium® IV microprocessor for data acquisition. Gemini-NX Column C18 (Phenomenex) (250 mm x 4.6 mm, i.d packed with 5µ particle size) was used for the chromatographic study. Mobile phase consisting of acetonitrile: 10 mM Tetra butyl ammonium hydrogen sulphate (60:40 v/v) at a flow rate of 1.0 ml/min (UV detection at 240 nm) (PDA) conditions were used for the elution of Fluoromethalone. The drug was subjected to various ICH described stress conditions including hydrolysis (acidic and alkaline) oxidation and thermal degradation. Fluoromethalone was found to degrade significantly in alkaline hydrolysis.

Results and Discussion: The proposed method was validated with respect to specificity, linearity, accuracy, precision, Limit of detection (LOD), Limit of Quantification (LOQ) and robustness as per ICH guidelines. The peak purity and resolution between drug and its degradants are satisfactory explaining the specificity of the method.

Conclusions: The developed method was successfully applied for the quality control of Fluorometholone in eye drops.

Keywords: Fluorometholone; Forced Degradation RP-HPLC; Eye Drops; Validation

Introduction

Fluorometholone (FRM) is also known as 6α-methyl - 9α-fluoro-11β, 17α - dihydroxy pregna-1, 4 - diene-3, 20-dione, is a synthetic glucocorticoid (Figure 1) which is used in the treatment of inflammatory eye diseases [1]. Fluorometholone (CAS No. 426 - 13 - 1) has molecular formula, C22H29FO4 and molecular weight 376.462 g/mol (pKa 12.65). It is used in the treatment of steroid-responsive inflammatory conditions of the palpebral and bulbar conjunctiva, cornea, and anterior segment of the eye. Corticosteroids such as Fluorometholone inhibit the inflammatory response to a variety of inciting agents and probably delay or slow healing. They inhibit the edema, fibrin deposition, capillary dilation, leukocyte migration, capillary proliferation, fibroblast proliferation, deposition of collagen, and scar formation associated with inflammation. Fluoromethalone was quantified by different techniques such as LC-MS [2], HPLC [3-5] and spectrophotometry [6-9] and in the present study the authors performed a reverse phase stability indicating liquid chromatographic technique for the ophthalmic preparations i.e. eye drops, and the method was validated.

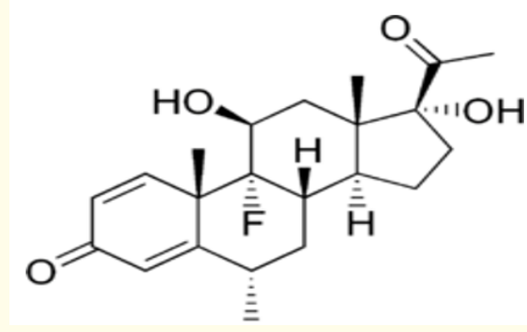

Figure 1: Chemical structures of fluorometholone (FRM).

Materials and Methods

Chemicals and reagents

FRM reference standard was kindly provided by Micro labs. Two Commercially available eye drops contained (brand1 and brand 2) were labeled to contain 1 mg/ml. the HYPLC Grade Aceto-

nitrile was purchased from Merck (Darmstdt, Germany), All other chemicals and solvents used were of analytical grade. HPLC purity water was prepared by using a Milli Q Ro System (Millipore USA).

Instrumentation and Chromatographic Conditions

Waters Alliance binary gradient HPLC system (Waters Corporation, MA, USA) equipped with a Waters 2695 separations module and Waters 2695 PDA detector was used for the present study. Empower pro-software was operated on a Pentium® IV microprocessor for data acquisition. Gemini-NX Column C18 (Phenomenex) (250 mm x 4.6 mm, i.d packed with 5µ particle size) was used. Chromatographic separation was carried out under isocratic mode with acetonitrile: 10mM Tetra butyl ammonium hydrogen sulphate (TBAHS) (60:40, v/v) at a flow rate of 1.0ml/min. The mobile phase was filtered through 0.45µ nylon membrane and degassed prior to use. The wavelength was monitored at 240 nm.

Preparation of Stock and Standard solutions

A standard stock solution of Fluorometholone (10 mg/ml) was prepared in HPLC grade methanol. Aliquots of stock solutions were transferred and diluted with mobile phase to yield concentrations of 25, 40, 60, 80, 120 and145 µg/ml.

Linearity and Range

A series of solutions (25 - 145 µg/ml) were prepared from the FRM stock solution with mobile phase and 10 µL of each of these solutions were injected in to the HPLC system. The peak area of FRM from the chromatograms were noted and a calibration curve was drawn by taking the concentration of FRM solutions on the x-axis and the corresponding mean peak area values on the y-axis. The limit of quantification and limit of detection measured as described in ICH guidelines Q2 (R1).

Precision

The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of FRM at three concentration levels (40, 80 and 120 µg/ml) against a qualified reference standard. The % RSD of three obtained assay values at three different concentration levels was calculated. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (40, 80 and 120 µg/ml) and the % RSD was calculated.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of FRM in the drug product. The study was carried out in triplicate at 40, 80 and 120 µg/ml. The percentage recovery in each case was calculated.

Robustness

The robustness of the assay method was established by introducing deliberate changes in the HPLC conditions which included wavelength (238 and 242 nm), percentage of Acetonitrile in the mobile phase (57 and 63%) and flow rate (0.9 and 1.1 ml/min). Robustness of the method was studied using six replicates at a concentration level of 80 µg/ml of FRM.

Assay of Fluorometholone

Fluorometholone is marketed with brand names Flurisone (Label claim: 0.1% and 0.25%) (Micro Vision), Biflace eye drops (Alembic Pharma), FML Forte (Allergan India Ltd) and F.M.L. Fluorometholone eye drops were procured from the pharmacy and extracted with acetonitrile and then diluted with mobile phase. The resulting solutions were filtered and then injected in to the system to record the chromatogram from which the peak area was noted. The percentage purity of the drug was determined from the linear regression equation and the results were tabulated.

Stress degradation studies of Fluorometholone

Stress degradation studies of Fluorometholone were executed in accordance with ICH guidelines as indicated below. 0.8 mg/ml of Fluorometholone was used for the degradation studies and all the solutions were filtered through 0.45µ nylon filter.

Acidic and alkaline hydrolytic degradation studies

Fluorometholone solutions were treated separately with 0.5N-5N HCl and 0.5N-5N NaOH for acidic and alkaline hydrolysis respectively at 80°C for 2 hrs, cooled and then neutralized. Later the resulting solutions were made up with mobile phase, filtered and then injected to determine the percentage of degradation of drug from the resulting chromatogram.

Oxidative degradation studies

Fluorometholone solutions were treated with 6% v/v H2O2 at 80°C for 60 minutes and then cooled and then diluted with mobile phase.

Photolytic degradation studies

Photo degradation studies were carried out by exposing the Fluorometholone solutions to UV light in UV Chamber at wavelength 254 nm for 8 hrs.

Thermal degradation studies

Thermal degradation studies were performed by exposing Fluorometholone solutions to heat at 80°C for 8 hrs on water bath.

Results and Discussion

Optimization of the HPLC Condition

Various aqueous solutions of inorganic salts with different pH and organic solvents (methanol and acetonitrile) were investi-

gated and system suitability parameters were performed during the development and optimization of the method. However best separation of drug and its degradant products was achieved only when 10 mM TBAHS was incorporated in the mobile phase. The optimized condition was reached when isocratic mode was opted with Acetonitrile: TBAHS 60:40, v/v mixture with flow rate 1ml/min. The characteristic overlay chromatogram of Fluorometholone was shown in figure 2a. Table 1 describes clearly the advantages of the present method with the previously published analytical methods in the literature.

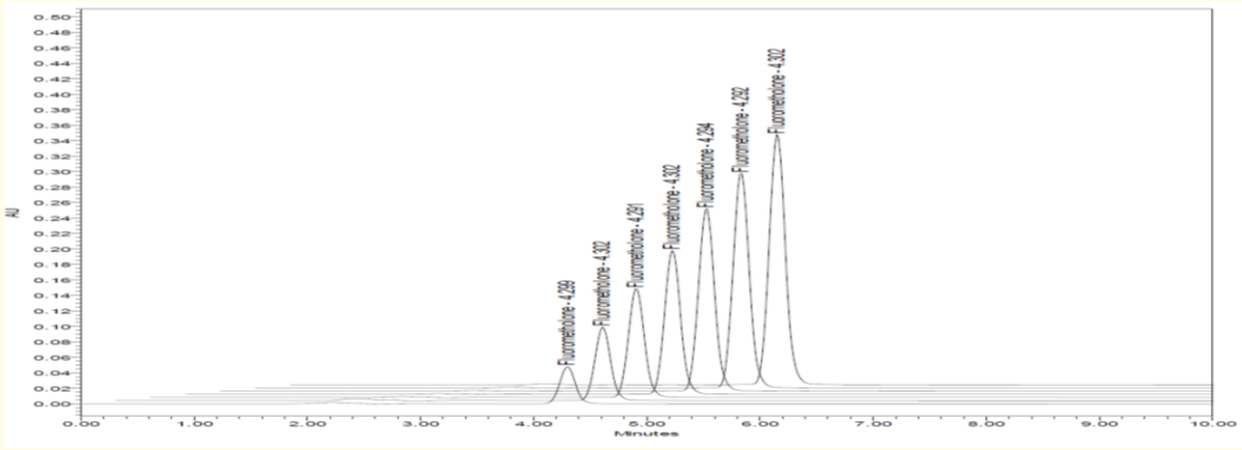

Figure 2a: Typical chromatograms of fluorometholone (Linearity).

Method / Reagents/Mobile phase (v/v)	Linearity (µg/mL)	Comments	Ref
Acetonitrile: methanol: 0.05 M potassium di hydrogen phosphate (0.1% trimethylamine); (pH 2.5, adjusted with ortho phosphoric acid) (20: 30: 50, v/v)	0.01 - 50	Combination with sodium cromoglycate HPTLC and HPLC	2
Methanol: water (58: 42)	0.25 - 2.0	HPLC	3
0.1% Acetic Acid: Methanol (20:80)	0.5 - 100	Stability indicating (PDA)	4
Methanol: Acetonitrile: water (50: 50: 10) Methanol	-	HPLC (Internal standard lidocaine) Derivative Spectroscopy	5
3-Hydroxy 4- (1-Azo-2,7-Dihydroxy) Naphthalene Sulfonic Acid	-	Spectrophotometry	6
1,4-dihydrazinophthalazine	Up to 12.0	Spectrophotometry	7
A) Methanol B) Octane Sulfonic Acid pH 3.0	0.1 - 80	Spectrophotometry	8
Absorptivity factor method Absorption factor method Mean centering of ratio spectra method Chloroform: Methanol: Toluene: Triethylamine (5:2:4:1, v/v/v/v)	4 - 16 4 - 16 2 - 16 0.2 - 1.6	Combination with sodium cromoglycate Spectrophotometry TLC-spectrodensitometry	9
Acetonitrile: 10 mM Tetra butyl ammonium hydrogen sulphate (60:40)	25 - 145	Stability indicating (PDA) Wide linearity range	Present method

Table 1: Scanning of published methods in the literature with the present method.

Validation of the method

The method was validated for system suitability, linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, solution stability and robustness (ICH, 2005).

Linearity and Range

Linearity was established by least squares linear regression analysis of calibration curve and evaluated by a determination coefficient (R^2). Standard solutions at different concentrations ranging from 25 - 145 µg/ml (Table 2) were prepared and analyzed in triplicates to prove the linearity of system. Mean peak area of FRM solution was plotted against their respective concentrations. The calibration curve has shown good linearity with linear regression equation y = 0.22328 x +14818 (R^2 = 0.999). The LOD and LOQ concentrations of FRM were found to be 0.01% and 0.05% respectively.

Conc. (µg/ml)	*Mean Peak Area
0	0
25	559331
40	922096
65	1484236
80	1812567
105	2341899
120	2726309
145	3222354

Table 2: Linearity of fluorometholone.

*Mean of three replicates.

Assay of Fluorometholone

The proposed method was applied to the determination of FRM in its eye drops formulations (Table 3) available and the % recovery was found to be 99.25 - 99.89% (Figure 2b and Figure 2c).

Formulation	Labelled claim (%)	Amount found (%)	Recovery* (%)
Brand I	0.1	0.09925	99.25
Brand II	0.1	0.09989	99.89

Table 3: Analysis of Fluorometholone in ophthalmic formulation.

* Mean of three replicates.

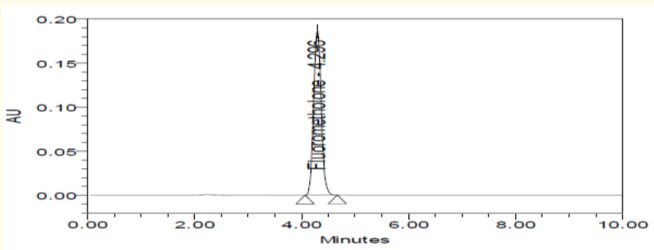

Figure 2b: Typical chromatograms of fluorometholone (Brand I).

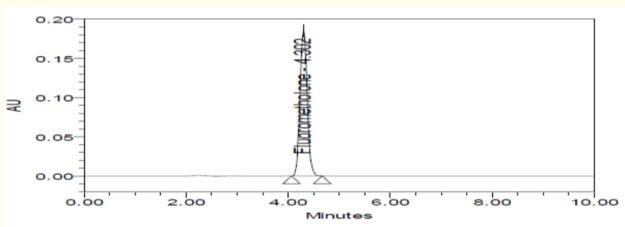

Figure 2c: Typical chromatograms of fluorometholone (Brand II).

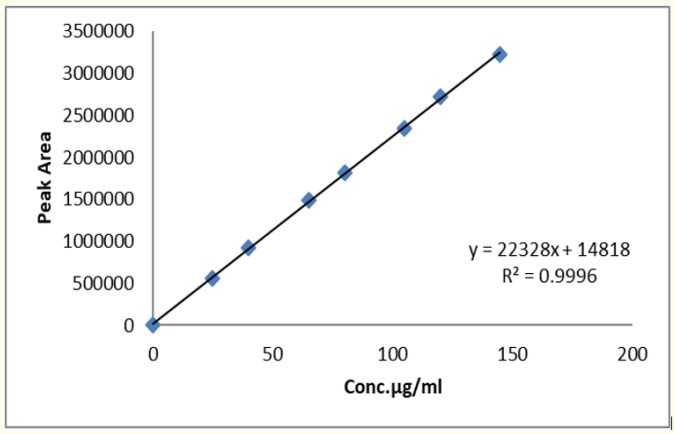

Figure 3: Calibration curve of fluorometholone.

Precision

The intra-day (Table 4a) precision of the method was determined by assaying three samples of each at three different concentration levels (40, 80 and 120 µg/ml) on the same day. The inter-day (Table 4b) precision was calculated by assaying three samples of each at three different concentration levels (40, 80 and 120 µg/ml) on three different days. The % RSD for intra-day precision was found to be 0.18 - 0.95 whereas the inter-day precision was found to be 0.27 - 0.84.

Conc. (µg/ml)	*Mean Peak Area	Statistical Analysis
		*Mean ± SD (% RSD)
40	942991	941724 ± 3077.737 (0.33)
40	943966	
40	938215	
80	1855176	1857480 ± 3283.523 (0.18)
80	1861240	
80	1856025	
120	2808504	2800095 ± 26646.363 (0.95)
120	2821523	
120	2770259	

Table 4a: Intraday precision study of Fluorometholone**.**

Conc. (µg/ml)	*Mean Peak Area			*Mean ± SD (%RSD)
	Day1	Day2	Day3	
40	952356	956898	956841	955365 ± 2606.026 (0.27)
80	1824563	1812547	1802365	1813158 ± 11111.620 (0.61)
120	2853125	2805611	2832564	2830433 ± 23828.5512 (0.84)

Table 4b: Inter day precision study of Fluorometholone.

Accuracy

The accuracy was evaluated by the recovery studies. A series of known amount of FRM standard was spiked in to placebo, mixed and the drug was extracted and diluted to yield three concentrations levels 50, 100 and 150% of the analytical method concentration. The accuracy data was presented in table 5. The mean recoveries ranged from 98.8 - 100.1% with % RSD < 2%. The result is found to be satisfactory for intended purpose and is adequate for routine analysis.

Conc. Level	Amount added (µg/ml)	Amount Recovered (µg/ml)	%Recovery	Mean
50%	60.19	59.25	98.44	98.8
	60.19	59.11	98.21	
	60.19	59.98	99.65	
100%	80.22	80.1	99.85	99.9
	80.22	80.24	100.02	
	80.22	79.98	99.70	
150%	100.14	100.55	100.41	100.1
	100.14	99.88	99.74	
	100.14	100.24	100.10	

Table 5: Accuracy study of Fluorometholone.

Robustness

The robustness was investigated by achieving deliberate changes in flow rate, detection wavelength, mobile phase composition etc., the system suitability parameters remain unaffected over deliberate small changes in the chromatographic system illustrating that method was robust over an acceptable working range of its HPLC Parameters. The % RSD of the FRM assay was 0.4% (< 1%).

Solution stability and mobile phase stability

The solution stability of FRM in the assay method was carried out by leaving both the sample and standard solutions at room temperature for 48h. The same sample solutions were analyzed at 12h intervals over the study period. The mobile phase stability was also assessed by analyzing freshly prepared reference standard solutions at 12h intervals up to 48h against mobile phase. The prepared mobile phase remained constant during the study period. The % RSD of the FRM assay was 0.4% (< 1%) for the mobile phase and solution stability experiments.

Forced Degradation Studies

The results obtained from the forced degradations studies were summarized in table 6. The drug is found to be more sensitive towards alkaline degradation circumstances. The system suitability parameter is within the acceptable criteria. The chromatograms and the corresponding purity plots obtained during the forced degradation studies were given in figure 4 and 5.

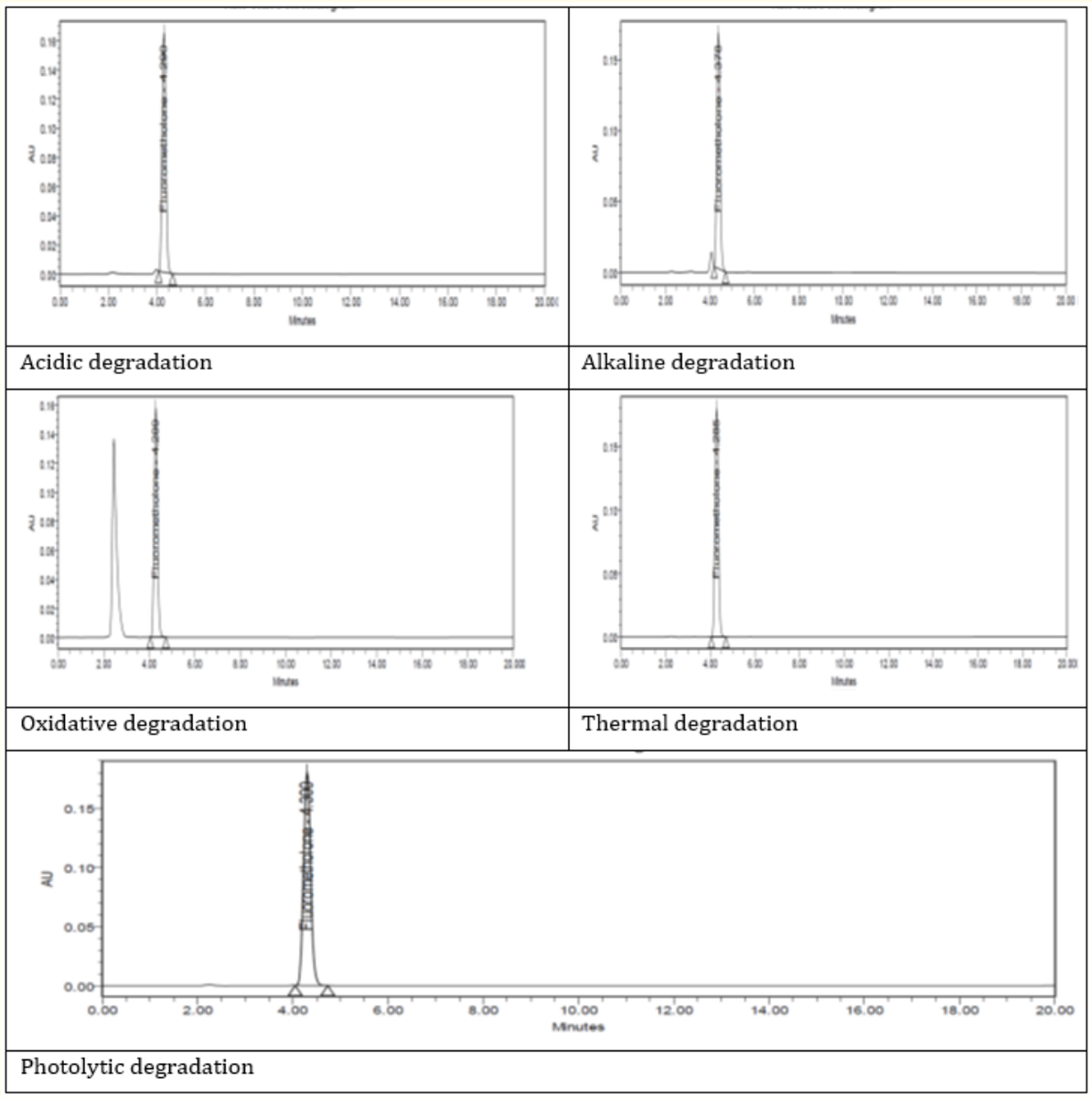

Figure 4: Chromatograms of Fluorometholone during stress degradation studies.

Stress Condition	Time (Hrs)	Purity Angle	Purity Threshold	% of Degradation
Acidic Hydrolysis (5N HCl (5ml) at 80°C)	2	0.048	0.212	5.7
Alkaline Hydrolysis (0.5N NaOH (5ml) at 80°C)	2	0.029	0.208	33.52
Oxidative degradation (6% H_2O_2 (5ml) at 80°C)	1	0.029	0.213	11.93
Thermal degradation (80°C)	8	0.023	0.210	0.06
Photo Stability	12	0.030	0.213	0.04

Table 6: Summary of forced degradation studies.

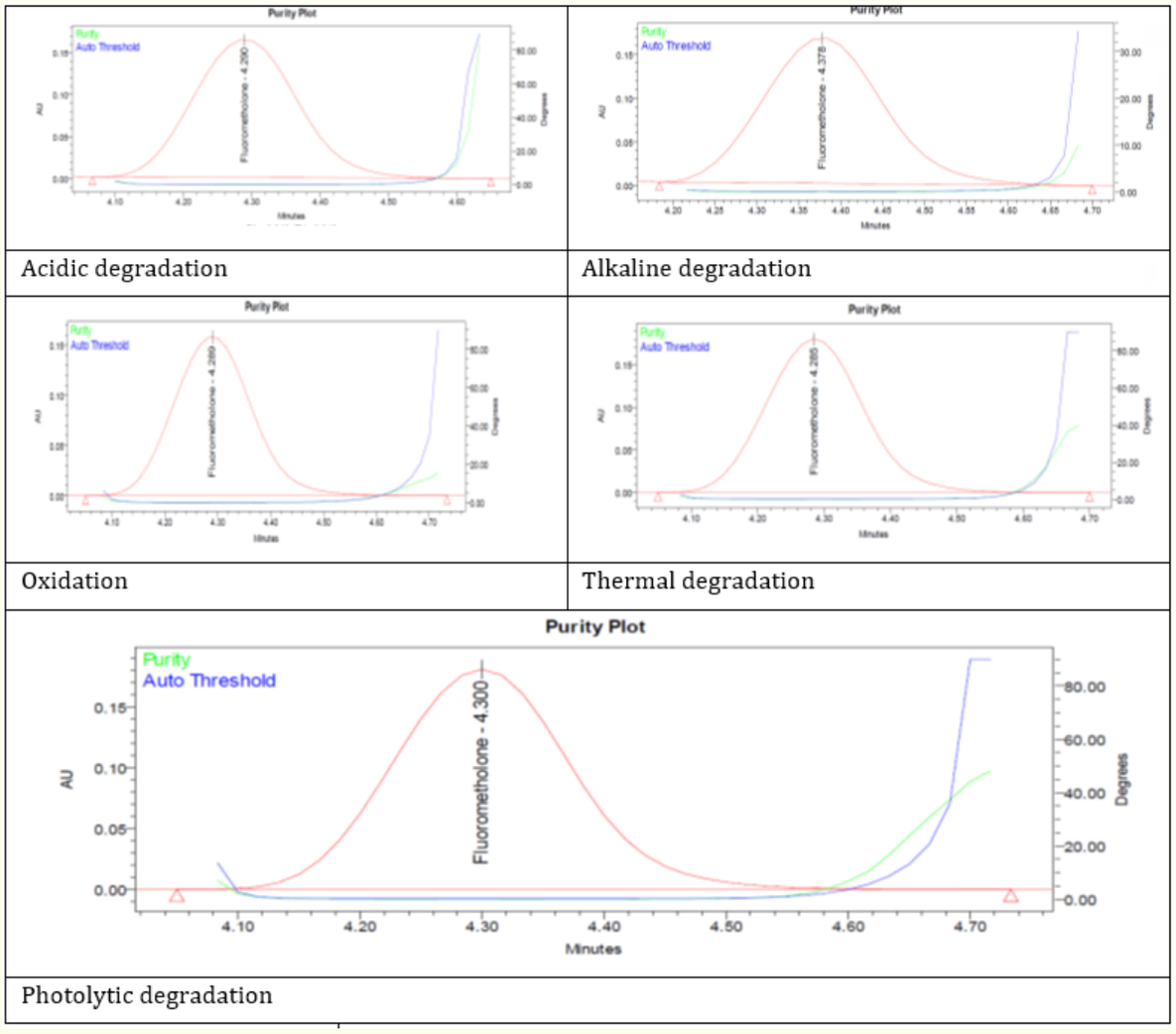

Figure 5: Purity plots of Fluorometholone.

Conclusion

The proposed stability-indicating HPLC method was validated as per ICH guidelines and applied for the determination of Fluorometholone in pharmaceutical dosage forms and can be successfully applied to perform long-term and accelerated stability studies of Fluorometholone formulations. It was observed that Fluorometholone is more sensitive towards alkaline degradation in comparison to others during the stress degradation studies.

Acknowledgement

The authors are grateful to Micro labs ltd., for providing the gift samples of Fluorometholone. There is no conflict of interest.

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