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Development and validation of an analytical method for the simultaneous quantification of Moxifloxacin and Bromofenac opthalmic solution by using RP-HPLC

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ABSTRACT

A new precise, accurate, rapid method has been developed for the simultaneous estimation of Bromofenac and moxifloxacin in pharmaceutical dosage form by RP-HPLC. The method was optimised with Mobile phase Phosphate buffer: Acetonitrile (40:60), Column Hypersil BDS,C18 flow rate 0.1µml, detection wave length at 275 nm .Retention time for bromofenac & moxifloxacin was found to be 2.3min & 3.6min with the above conditions. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity studies were carried out between 22.5µg/ml-135lµg/ml and 125µg/ml-750µg/ml levels of Bromofenac and moxifloxacin , R^2 value was found to be 0.999. Accuracy was determined at three different levels 50%, 100%, 150% and %mean recovery studies were found to be 99.83, 100.9, 99.82 and 99.70, 99.66, 100.39 for Bromofenac and moxifloxacin respectively.The method is precise as the %RSD values of peak areas for Moxifloxacin and Bromofenac were found to be 0.39 and 0.40 respectively which are well within the acceptance criteria limit (RSD ≤ 2).The method was subjected to degradation studies through acid, base, photo stability degradations. In all the cases the % degradation is very less, the purity angle was found to be less than threshold and there are no co-eluting peaks near the R_t of the drugs. So we assume that the method is specific and stable one .Hence we conclude that the method is simple, specific, rapid, linear, accurate, precise, robust, stable and economical. Therefore, this method can be used for the estimation of bromofenac & moxifloxacin in Pharmaceutical dosage form for routine analysis purpose.

Key words: Moxifloxacin, Bromofenac, RP-HPLC, Degradation studies.

INTRODUCTION

Bromofenac is a non-steroidal anti-inflammatory drug (NSAID) marketed in the US as an ophthalmic solution (current brand names Prolensa and Bromday, prior formulation brand name Xibrom, which has since been discontinued.) by ISTA Pharmaceuticals for short-term, local use. Prolensa and Bromday are the once-daily formulation of bromfenac, while Xibrom was approved for twice-daily administration. Bromfenac is indicated for the treatment of ocular inflammation and pain after cataract surgery, though it may be prescribed in an off-label manner by the physician.

For ophthalmic use, bromfenac has been prescribed more than 20,000,000 times across the world. As an eye drop, it has been available since 2000, starting in Japan where it was sold as Bronuck. It was first FDA approved for use in the United States in 2005, and it was marketed as Xibrom, twice-daily. In October 2010 Bromday received FDA approval as a new, once-daily formulation. More recently, in 2013, Prolensa has also been approved by the FDA. The bromfenac molecule will be marketed in Europe and other worldwide markets with agreements from Bausch & Lomb, Croma Pharma, and other companies.

Moxifloxacin is a synthetic fluoroquinolone antibacterial agent developed by Bayer AG (initially called BAY 12-8039). It is marketed worldwide (as the hydrochloride) under the brand names Avelox, Avalox, and Avelon for oral treatment. In most countries, the drug is also available in parenteral form for intravenous infusion. Moxifloxacin is also sold in an ophthalmic solution (eye drops) under the brand names Vigamox, and Moxeza for the treatment of conjunctivitis (pink eye). It's antibacterial spectrum includes enteric Gram-(-) rods (Escherichia coli, Proteus species, Klebsiella species), Haemophilus influenzae, atypical bacteria (Mycoplasma, Chlamydia, Legionella), and Streptococcus pneumoniae, and anaerobic bacteria. It differs from earlier antibacterials of the fluoroquinolone class such as levofloxacin and ciprofloxacin in having greater activity against Gram-(+) bacteria and anaerobes. Because of its potent activity against the common respiratory pathogen Streptococcus pneumoniae, it is considered a "respiratory quinolone."

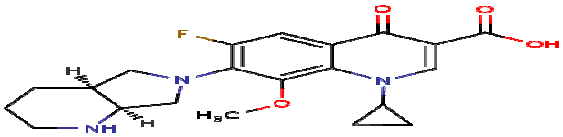


Fig 1: Moxifloxacin

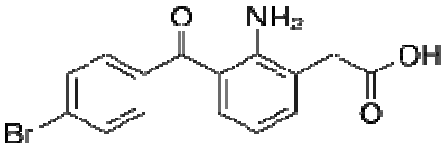


Fig 2 : Bromofenac

MATERIALS AND METHODS

Instruments

The present work utilized Shimadzu HPLC system with LC-solution software. A reverse phase column (make: Phenomenex, 250mm, 4.4mm, particle size 5µ) was used. UV-spectra were obtained from PG-T60 UV-.Visible Spectrophotometer

Chemicals and Solvents:

Water, Methanol, Acetonitrile belonging to HPLC grade and chemicals like Potassium dihydrogen ortho phosphate, ortho phosphoric acid, sodium dihydrogen phosphate of AR grade were purchased from Merk (India) Ltd.

Selection of Solvent

To develop a rugged and suitable method for quantitative determination of Moxifloxacin and Bromofenac, the analytical conditions were selected after the consideration of different parameters such as diluents, buffer, buffer concentration, and organic solvent for mobile phase, mobile phase composition and other chromatographic conditions.

Selection of method depends upon nature of the sample, its molecular weight and solubility. The Ophthalmic solution dispersed readily in solvent as Moxifloxacin and Bromofenac are freely soluble in water and acetonitrile. Moxifloxacin and Bromofenac can be easily extracted from the pharmaceutical dosage form by using of buffer and acetonitrile (40:60)

Selection of wavelength

A detection wavelength of 275nm was selected after scanning the standard solution over the range 200-400nm by use of the PDA detector. Detection at 275 nm resulted in good response Moxifloxacin and Bromofenac were injected with following chromatographic conditions.

Preparation of buffer:

Accurately weighed 2.72gm of potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 0.2ml of Triethylamine then PH adjusted to 3.8 with dil. Orthophosphoric acid solution.

Standard stock solution of Moxifloxacin

Accurately 50 mg of Moxifloxacin was weighed and transferred into a clean and dry 10 ml volumetric flask, dissolved with sufficient volume of diluent and sonicate for 5min.The volume made up to 10ml with diluent (5000 µg/ml).

Standard stock solution of Bromofenac:

Accurately 10 mg of Bromofenac was weighed and transferred into a clean and dry 10 ml volumetric flask, dissolved with sufficient volume of diluent and sonicate for 5 min.The volume made up to 100ml with diluent .

Chromatographic conditions:
A reverse phase HPLC column Hypersil BDS, C18, (250mm, 4.6mm, and particle size 5µ) was used for elution at ambient temperature. The mobile phase was pumped through the column at a flow rate of 1.0ml/min. The sample injection volume was 10 µl. The detector was set to a wavelength of 275 nm and the chromatographic run time was set to 7 minutes.

Method Development:
The method development was started with initial chromatographic conditions as stated above. Various compositions of phosphate buffer and acetonitrile were tested for better separation of the analytes. The method was optimised with Mobile phase, Phosphate buffer: Acetonitrile (40:60), Column Hypersil BDS,C18 flow rate 0.1µml, detection wave length at 275 nm .Retention time for bromofenac & moxifloxacin was found to be 2.3min & 3.6min with the above conditions. With this composition peaks for both the analytes were eluted with good resolution and the retention times and theoretical plates were also satisfactory. The chromatogram has passed the system suitability parameters and the retention times for Moxifloxacin and Bromofenac were found to be 2.3min & 3.6min respectively. The chromatogram is shown **in figure-3.**

Method validation:
The proposed method for the simultaneous estimation of Bromofenac and Moxifloxacin in combined as per. dosage form is validated ICH guidelines by the following parameters.

System Suitability:
Sample solution was injected three times as per the procedure and the chromatogram was recorded. System suitability parameters like tailing factor, theoretical plates and peak areas were checked. The results are given in **table n.o:1**

Linearity:
Aliquots of standard stock solutions of Bromofenac and Moxifloxacin were transferred into 10ml volumetric flasks and diluted up to the mark by diluents to achieve the concentrations of 62.5 to 375 µg/ml for Bromofenac and 25 to 150 µg/ml for Moxifloxacin. Each sample solution was injected into HPLC system and the peak areas were measured. A graph of peak areas vs concentrations was plotted and the r^2 values were calculated. The results were shown in **fig 4 & 5**, **table –2 & 3**.

Precision:
The intraday and interday precision of the proposed method was determined by analyzing the corresponding responses on the same day and on different days for replicate concentrations of standard solutions of Moxifloxacin and Bromofenac. The results were reported in terms of relative standard deviation (RSD). The results obtained are presented in and **table No: 4 & 5**

Accuracy
The accuracy of the proposed method was evaluated by recovery studies at various concentrations of Moxifloxacin and Bromofenac equivalent to 99.82 & 99.66%. The percentage recovery at each level was calculated and reported in **table – 6**

Specificity:
It is the ability of the method to measure the analyte of interest specifically in presence of matrix and other components. Samples of blank and placebo were injected as per the test procedure. The chromatograms of blank and placebo were represented as **Fig no: 6 & 7**.

Limit of Detection and Limit of Quantification:
The detection limit of an analytical method is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated**.**

$$\textbf{LOD} = \frac{\textbf{3.3}\times\textbf{Standard deviation}}{\textbf{Slope}}$$

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy.

$$\textbf{LOQ} = \frac{\textbf{10}\times\textbf{Standard deviation}}{\textbf{Slope}}$$

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

The slope S may be estimated from the calibration curve of the analyte.

The results were given in **table – 7.**

Robustness:
The robustness of the proposed method was determined by recording the chromatograms with small deliberate changes in parameters like flow rate, mobile phase etc.

Effect of variation of flow rate:
The flow rate is varied between 0.7-1.1ml/min from the optimized flow rate 1ml/min. Sample solutions were prepared and injected into HPLC system and the chromatograms are recorded.

Change in Mobile Phase:
Mobile phases Buffer and acetonitrile of 70:30 v/v and 80:20 v/v were selected. Working standard solutions (100µg/ml and 250µg/ml) were injected separately in chromatograph and chromatograms were recorded presented in **Table No: 8.**

The chromatograms for robustness studies are given in **fig no: 8,9,10 & 11**

RESULTS AND DISCUSSION

Once the initial conditions are set, at each trial a slight variation in mobile phase composition was made to overcome the demerits in the previous trial. Finally the method was optimized with mobile phase composition buffer: acetonitrile at a ratio 40: 60 %v/v. Chromatographic detection was done at 275 nm. The results are follows:

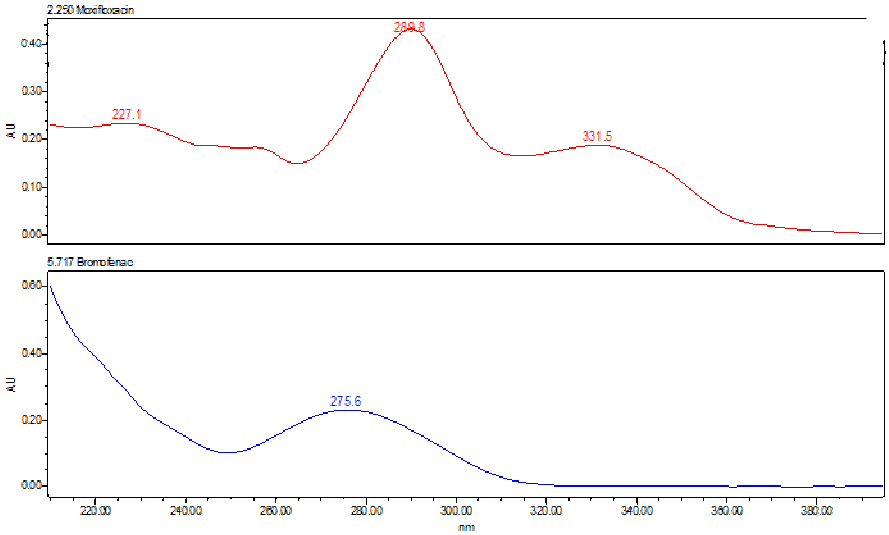


Fig 1: UV Spectrums of Bromofenac and Moxifloxacin

Method Validation:
The credibility of the proposed method was established by validation as per ICH guidelines. The method was validated through some parameters and the results are as follows:

System suitability:
The % RSD of retention time and peak areas of both the drugs was less than 2 and the other system suitability parameters were within the acceptable limits.

Table 1 System suitability parameters

Parameters	Moxifloxacin	Bromofenac	Acceptance criteria
Theoretical plates	3144	4344	More than 2000
Tailing factor	1.28	1.10	Less than 2
Retention time	2.31	3.68	More than 2

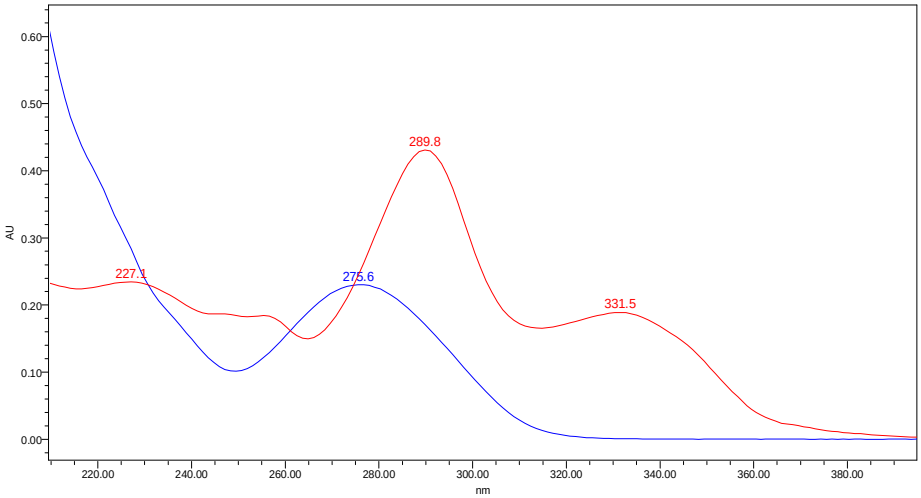


Fig: 2 UV-VIS overlay Spectrum of Bromofenac and Moxifloxacin

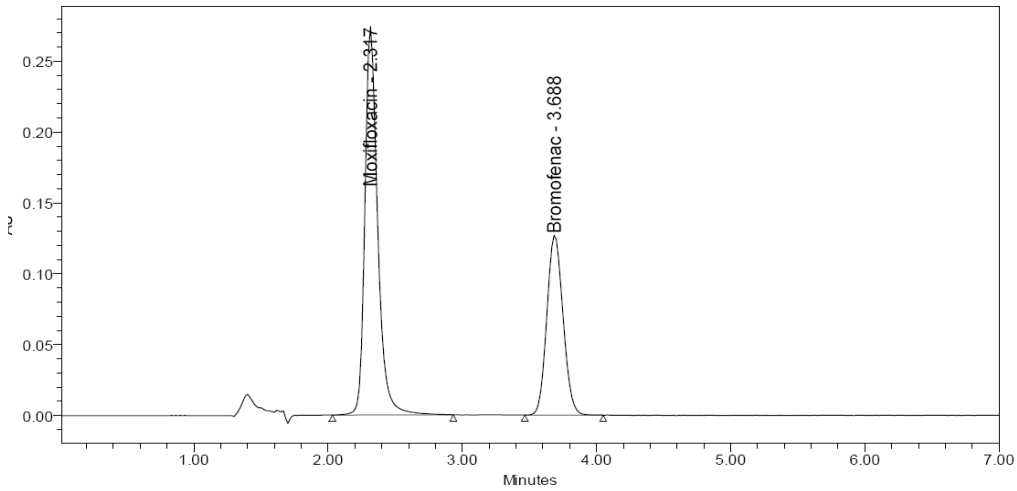


Fig no: 3 Chromatogram of optimized trial

Linearity:

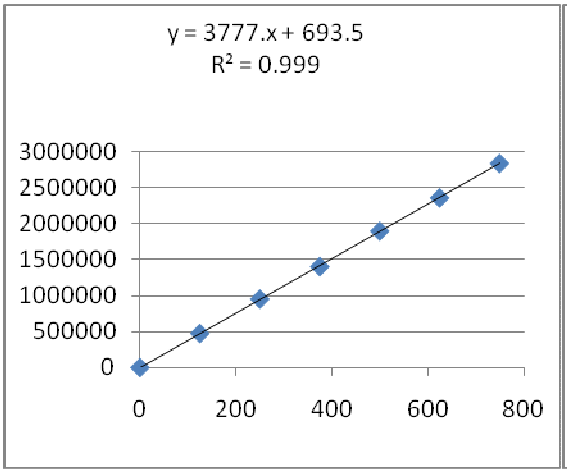
The linearity of the method was evaluated at various concentration levels equivalent to 125-750% for Moxifloxacin and 22.5-135% for Bromofenac. The results are as follows:

Table no: 2 Linearity data of Moxifloxacin and Bromofenac

S.No	Moxifloxacin(%)	Peak Area	Bromofenac(%)	Peak Area
1	125	474698	22.5	288521
2	250	952380	45	587843
3	375	1403009	67.5	872885
4	500	1895900	90	1134899
5	625	2358119	112.5	1447972

Table no: 3 Calibration parameters for Moxifloxacin and Bromofenac

Parameter	Moxifloxacin	Bromofenac
Slope	3777	12837
Intercept	693.5	1023
Correlation co-efficient	0.999	0.999



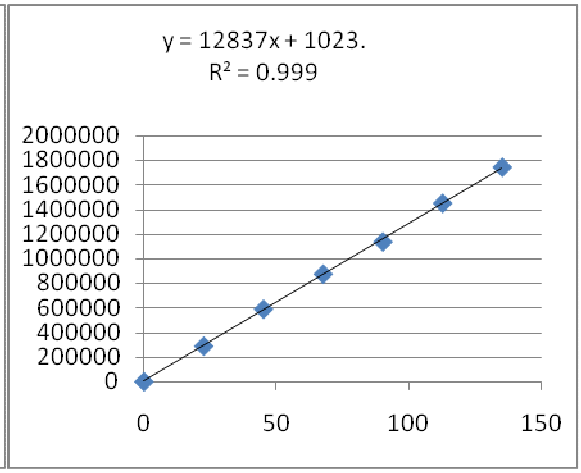


Fig :4 Calibration Curve Of Moxifloxacin Fig :5 Calibration Curve Of Bromofenac

Precision:

The sample solutions were injected for six times and peak areas from all six injections were measured in HPLC. The %RSD for the peak areas of six replicate injections were found to be within the specified limits. (%RSD<2).

Table no: 4 Intra-day precision results for Moxifloxacin and Bromofenac

Injection. No	Moxifloxacin		Bromofenac	
	Peak Area	Retention Time	Peak area	Retention Time
1	1807595	2.317	1075965	3.688
2	1822647	2.340	1085480	3.709
3	1838470	2.344	1078719	3.712
4	1810069	2.367	1092631	3.741
5	1822294	2.372	1078363	3.757
6	1839047	2.394	1085188	3.777
Avg	1823354		1082724	
SD	13425.3		6205.7	
%RSD	0.74		0.6	

Table no: 5 Inter-day precision results for Moxifloxacin and Bromofenac

Injection. No	Moxifloxacin		Bromofenac	
	Peak Area	Retention Time	Peak area	Retention Time
1	1841094	2.42	1104993	3.812
2	1853167	2.422	1094972	3.814
3	1848708	2.424	1093681	3.814
4	1847925	2.424	1101709	3.814
5	1838278	2.425	1097788	3.816
6	1857524	2.555	1095118	3.891
Avg	1847783		1098044	
SD	7208.9		4445.69	
%RSD	0.39		0.40	

Accuracy:

The accuracy of the proposed method was evaluated by recovery studies where the sample solutions are injected in triplicates at three different levels.

Table no: 6 Accuracy results of Moxifloxacin and Bromofenac

Level %	No	Recovery (%)	Mean recovery (%)	Recovery (%)	Mean recovery (%)
50%	**1**	**99.95**		**99.30**	
	2	**100.05**	**99.83**	**99.54**	**99.70**
	3	**99.47**		**100.24**	
100%	**1**	**100.46**		**99.49**	
	2	**99.67**	**100.9**	**100.23**	**99.66**
	3	**100.12**		**99.28**	
150%	**1**	**100.33**		**100.94**	
	2	**99.66**	**99.82**	**99.83**	**100.39**
	3	**99.48**		**100.40**	

The % recoveries are in the range 99.82-100.09%, and 99.66-100.39 % for Moxifloxacin and Bromofenac respectively.

Specificity:

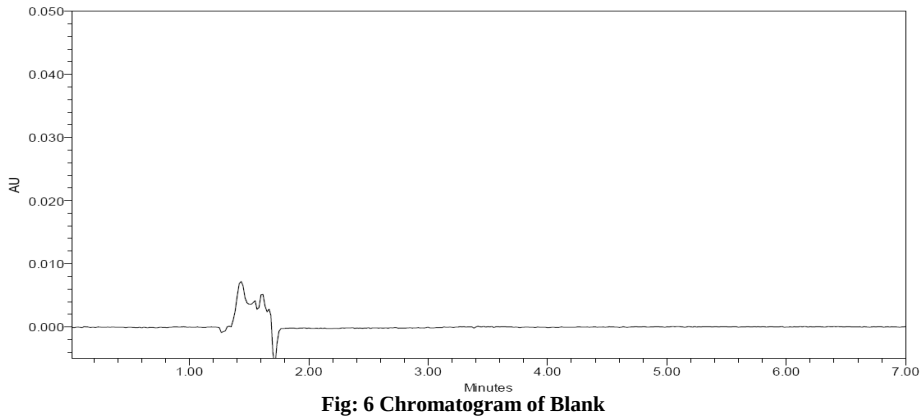


Fig: 6 Chromatogram of Blank

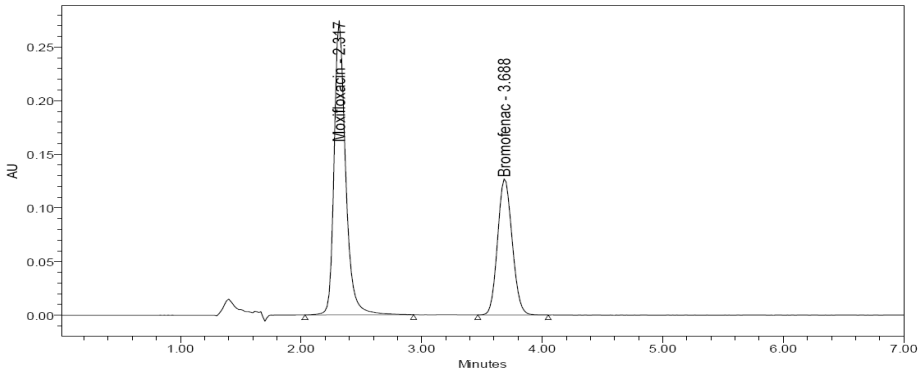


Fig: 7 Chromatogram of Moxifloxacin and Bromofenac standard

No peaks were observed near the retention times of Moxifloxacin and Bromofenac in the chromatogram of blank indicating no interference from mobile phase. Therefore the method is specific.

LOD & LOQ:

Limit of Detection and Limit of Quantification were calculated and reported in table-8.

Table no: 7 LOD & LOQ data of Moxifloxacin and Bromofenac

S.no	Parameter	Moxifloxacin	Bromofenac
1	LOD(µg/ml)	3.3 µg/ml	0.26 µg/ml
2	LOQ(µg/ml)	10.0 µg/ml	0.80 µg/ml

Robustness

The robustness of the method was evaluated by deliberate changes in flow rate and wavelength. The chromatograms are recorded and the parameters like efficiency and asymmetry was studied.

Effect of variation in flow rate

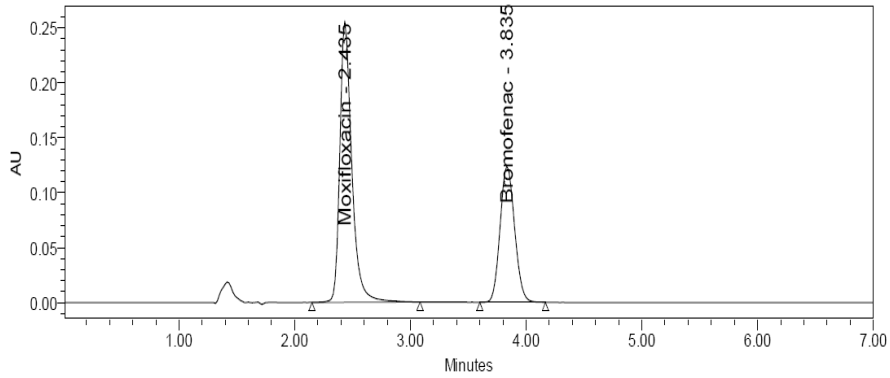


Fig:8 Chromatogram for Moxifloxacin and Bromofenac with decrease in flow rate (0.9ml/min)

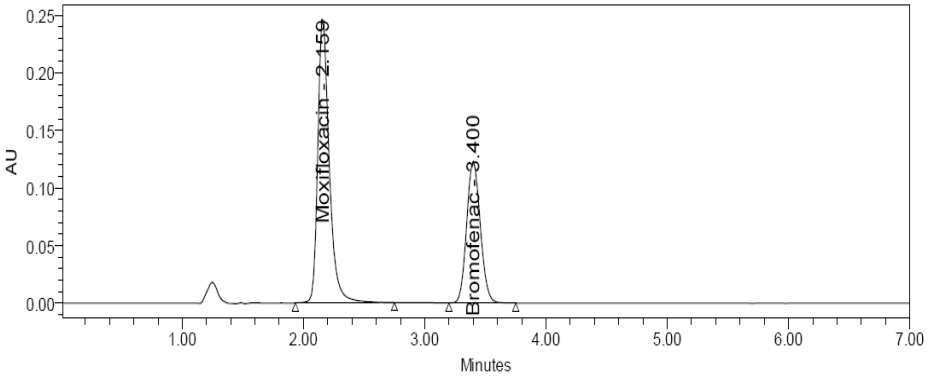


Fig: 9 Chromatogram for Moxifloxacin and Bromofenac with increase in flow rate (1.1ml/min)

Effect of variation in mobile phase:

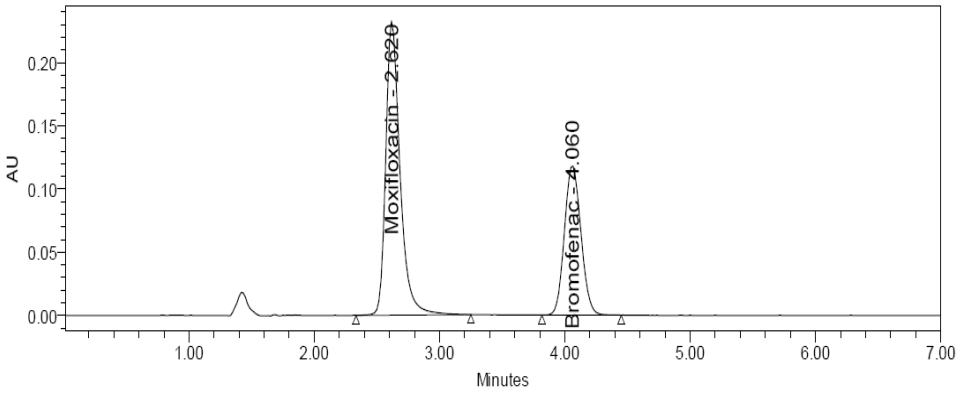


Fig:10 chromatogram for Moxifloxacin and Bromofenac with increase in organic phase(30:70v/v)

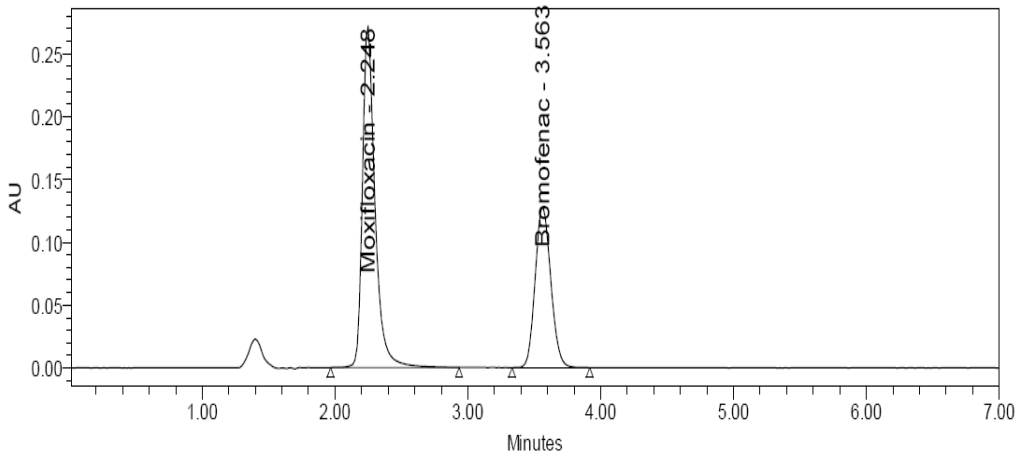


Fig:11 Chromatogram for Moxifloxacin and Bromofenac with decrease in organic phase (80:20 v/v)

Table 8: Results of Robustness

Parameters		Bromofenac		Moxifloxacin	
		Rt	PeakArea	Rt	Peak Area
	0.9ml/min	3.821	1080882	2.426	1830150
		3.835	1079136	2.435	1829119
Flow Rate	1.0ml/min	3.65	1080579	2.299	1820072
		3.654	1082865	2.301	1818489
	1.1ml/min	3.4	959906	2.159	1618595
		3.4	954646	2.159	1613085
	70:30	3.562	1042934	2.248	1775983
		3.563	1042687	2.251	1769280
	75:25	3.657	1080503	2.302	1823529
Mobile Phase		3.662	1080544	2.304	1817908
	80:20	4.06	1083281	2.62	1836424
		4.064	1086615	2.622	1836632

FORCED DEGRADATION

The Data for Forced degradation are tabulated in **Table 9**. There was no interference of any peak at the retention time of analyte peaks from blank and placebo, Peak purity of all the treated samples was well within the limits. From this it has been concluded that the proposed method is specific and stability indicating for the estimation of Bromofenac and Moxifloxacin in ophthalmic solution.

Oxidation:

To 1 ml of stock solution of Moxifloxacin and Bromofenac, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c.

For HPLC study, the resultant solution was diluted to obtain 500µg/ml&90µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The results are present in **fig no: 14**

Acid Degradation Studies:

To 1 ml of stock ssolution Moxifloxacin and Bromofenac, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain 500µg/ml&90µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.The results are present in **fig no: 12**.

Alkali Degradation Studies:

To 1 ml of stock solution Moxifloxacin and Bromofenac, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 500µg/ml & 90 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The results are present in **fig no: 13**.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105^0c for 6 h to study dry heat degradation. For HPLC study,

the resultant solution was diluted to 500µg/ml & 90µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** The results are present in **fig no: 15**.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 500µg/ml&90µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The results are present in **fig no: 16.**

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 500µg/ml&90µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** The results are presented in the **fig no: 17**

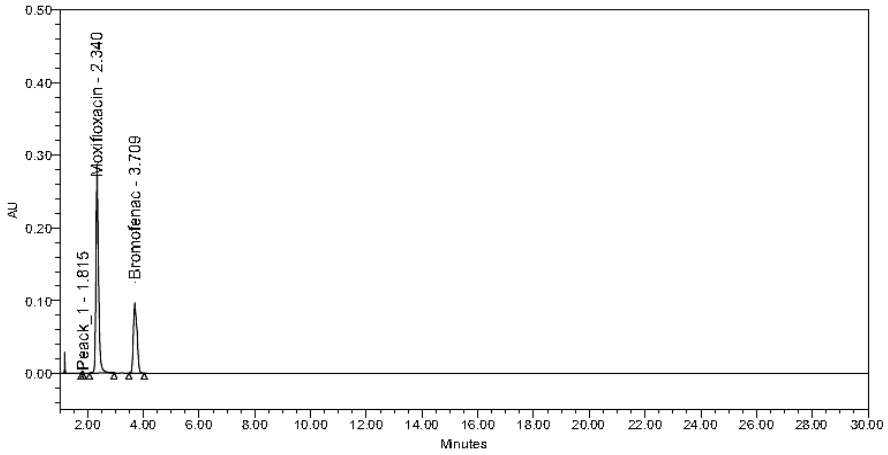


Fig: 12 Chromatogarm of Acid Sample (2 N HCl):

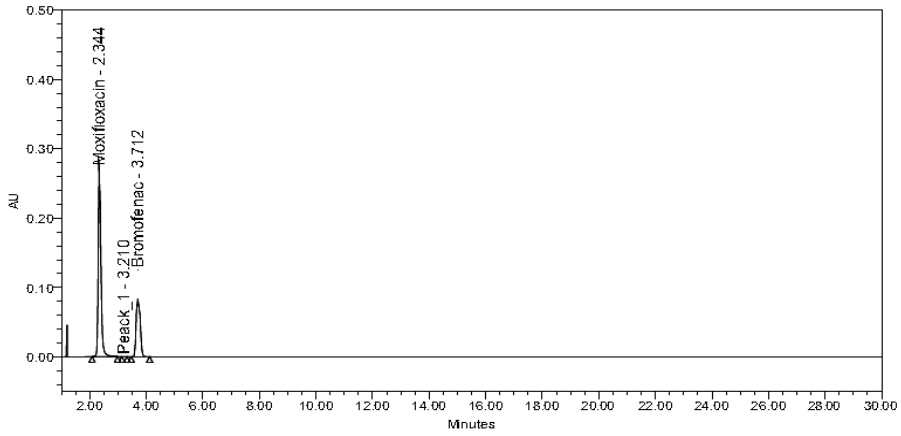


Fig: 13 Chromatogram of Base Sample (2 N NaoH):

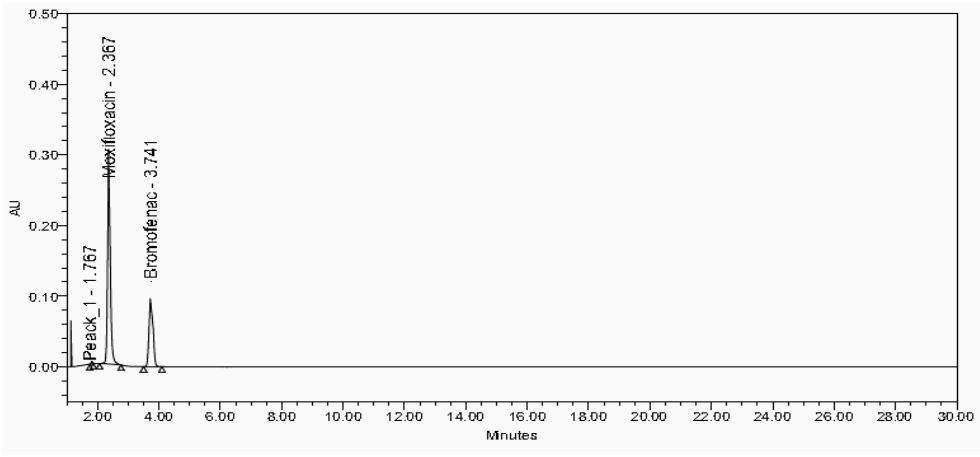


Fig: 14 Chromatogram of Peroxide Sample

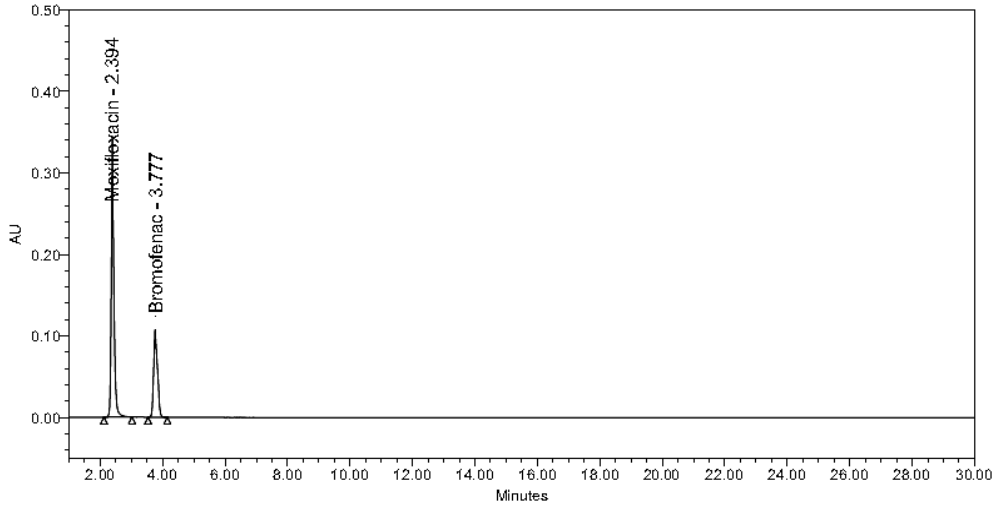


Fig: 15 Chromatogram of Thermal Sample (105^{0}c for 6 h):

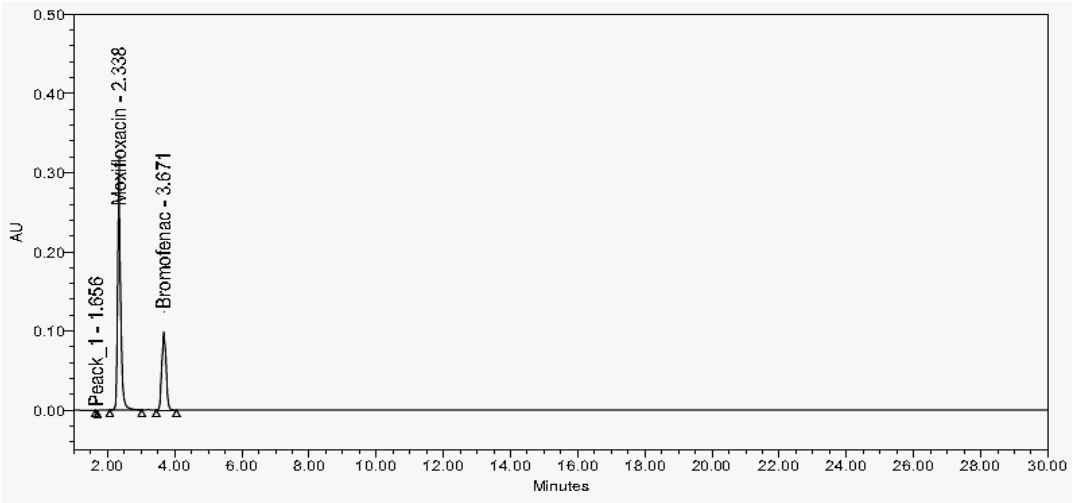


Fig: 16 Chromatogram of UV Sample (UV Chamber for 7days)

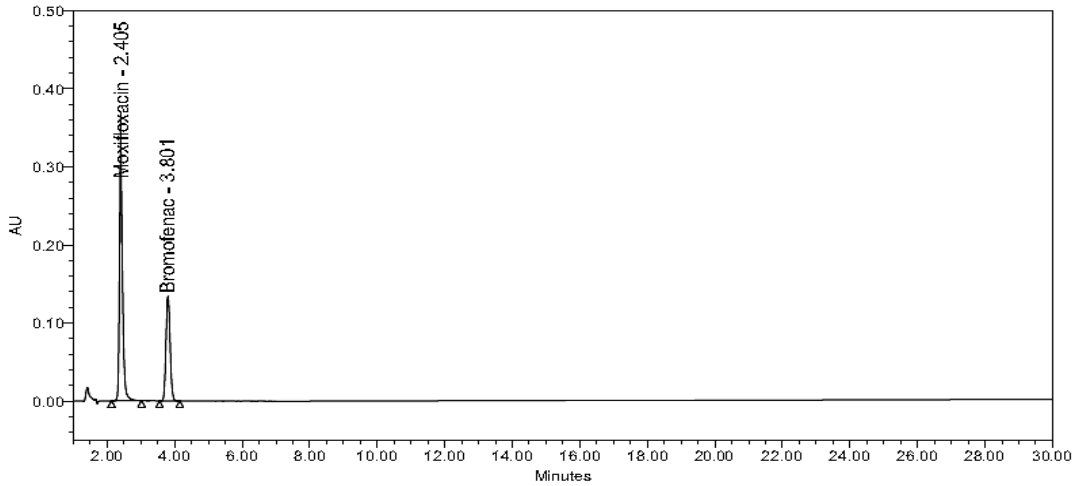


Fig: 17 Chromatogram of Neutral Sample (Reflux for 6 hrs at 60°C)

Table 9: Data of forced degradation

S.No	Sample condition	Analytes	% ASSAY	% Degradation	Purity angle	Purity threshold
1	Untreated sample	Moxifloxacin	99.87	--	0.151	0.727
		Bromofenac	99.84	--	0.251	0.617
2	Peroxide treated	Moxifloxacin	94.40	5.47	0.067	0.278
		Bromofenac	94.40	5.44	0.089	0.139
3	Acid treated	Moxifloxacin	92.47	7.40	0.151	0.727
		Bromofenac	92.34	7.50	0351	0.424
4	Alkali treated	Moxifloxacin	93.90	5.97	0.078	0.281
		Bromofenac	93.19	6.65	0.087	0.451
5	Thermal /Dry heat exposed	Moxifloxacin	95.61	4.26	0.2	0.418
		Bromofenac	95.61	4.23	0.378	0.518
6	Photolytic degradation	Moxifloxacin	98.36	1.51	0.089	0.430
		Bromofenac	98.36	1.48	0.173	0.321

CONCLUSION

An attempt was made to develop a simple, accurate, economical and precise method for the routine analysis of Moxifloxacin & Brmofenac in Occular Dosage form. Finally the method was optimised with Mobile phase Phosphate buffer: Acetonitrile (40:60). The proposed method was validated for system suitability, linearity, precision, accuracy, specificity, robustness, LOD and LOQ. From the validation results it has been evident that the method was linear, precise, accurate, sensitive and robust. The method was subjected to degradation studies through acid, base, photo stability degradations. In each case the % degradation is less, the purity angle was less than threshold and no co-eluting peaks near the R_t of the analytes. Therefore the proposed method could be a good approach for obtaining reliable results and suitable for the routine analysis of Moxifloxacin and Bromofenac in Bulk drug and ophthalmic dosage forms.

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