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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BIMATOPROST AND TIMOLOL MALEATE

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ABSTRACT

A simple, precise, specific, accurate and robust Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method has been developed & validated as per ICH guideline Q2 R(1) for the simultaneous determination of Bimatoprost and Timolol Maleate. Chromatographic separation of these two drugs were carried out on C_{18} column of Inertsil (250 x 4.6 mm, 5 µm) as stationary phase with a mobile phase consisting of Phosphate buffer (pH 6.0 adjusted with 1 N NaOH): ACN (60:40 %v/v) at a flow rate of 1ml / min and UV detection at 210 nm. The retention time of Bimatoprost and Timolol Maleate were 6.357 min and 2.203 respectively. The proposed method was validated for specificity, linearity, accuracy, precision, LOD & LOQ. The describe method was linear over a concentration range of 5-15 µg/ml, 25-75 µg/ml for Bimatoprost and Timolol Maleate respectively. The % recoveries of Bimatoprost and Timolol Maleate were found 99.667% - 101.292% and 100.266% - 101.763% respectively. The optimized and validated method can be used for estimation of Bimatoprost and Timolol Maleate in bulk and also in finished product.

KEYWORDS: Bimatoprost, Timolol Maleate, RP-HPLC, Validation, Finished product.

INTRODUCTION

Glaucoma[1] is a group of diseases characterized by a progressive form of optic nerve damage and raised intra ocular pressure (IOP). The increased pressure, called intraocular pressure, can damage the optic nerve which transmits images to your brain. If the damage continues, glaucoma can lead to permanent vision loss.

Chemically Bimatoprost[2] ((Z)-7-[(1R, 2R, 3R, 5S)-3,5-dihydroxy-2-[(E,3S)-3-hydroxy-5-phenylpent-1-enyl]cyclopentyl]-N-ethylhept-5-enamide) is synthetic prostamide and structural prostaglandin analogue with ocular hypotensive activity. It mimics the effects of the endogenous prostamides and reduces intraocular pressure by increasing outflow of aqueous humor. Timolol Maleate[3] ((2S)-1-(tert-butylamino)-3-{[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy}propan-2-ol; (2Z)-but-2-enedioic acid) is β-blocker. It was first β-blocker to be used as an antiglaucoma agent. It reduces formation of aqueous humor by reduction of blood flow to the ciliary processes and cAMP synthesis and at the end reduces the IOP.

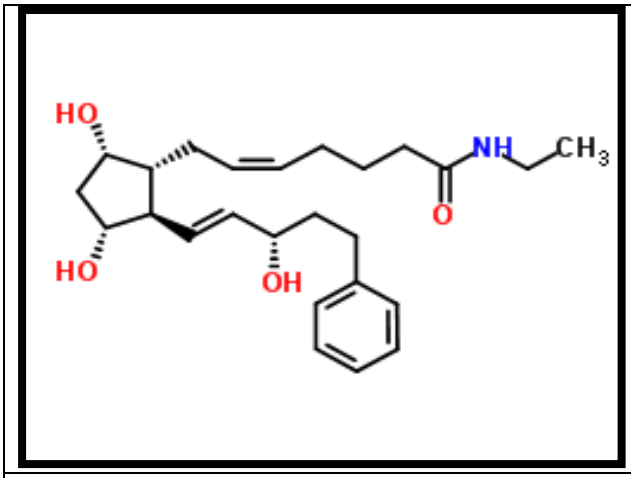

Figure 1: Structure of Bimatoprost.

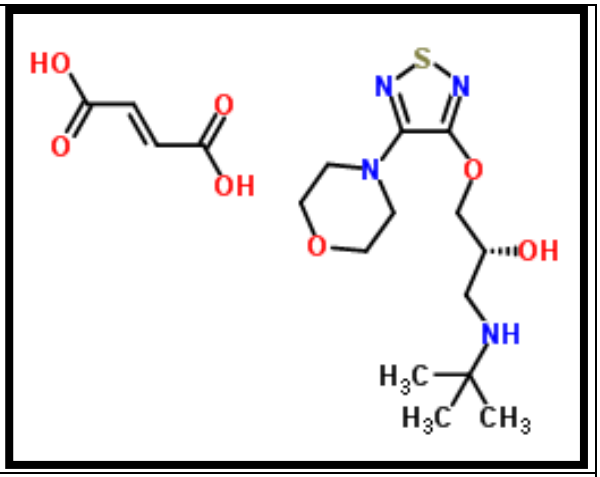

Figure 2: Structure of Timolol Maleate.

Based on Literature review, numbers of analytical methods (like UV spectrophotometer, Chromatography)[4-9] are available for estimation of both the drugs (Bimatoprost & Timolol Maleate) either alone or in combination with other drugs. But there is no analytical method for siultaneous estimation of both drugs by HPLC**.** In the view of the need for a suitable method for routine analysis in combined Formulations, attempts are being to develop simple, precise and accurate analytical method for simultaneous estimation of Bimatoprost and Timolol Maleate in eye drops.

MATERIALS AND METHOD

Reagents and Chemicals

Bimatoprost and Timolol Maleate were obtained as gift samples from Analytical research lab Ahmedabad. HPLC grade Acetonitrile, methanol, Water and Phosphoric acid; Sodium hydroxide analytical grade was obtained from SD Fine Chemical Ltd. Commercially available ophthalmic solution claimed to contain 0.03% w/v of Bimatoprost and 0.5% w/v of Timolol Maleate (Careprost Plus) was procured from local market.

Instrument and Chromatographic Conditions

Young lin HPLC system was used for method development and validation. Data acquisition was performed on YL-Clarity software. The separation were achieved on Inertsil ODS C18 (250 × 4.6 mm, 5µm) column. The column was maintained at room temperature and the eluent was monitored at 210nm using PDA detector. The mixture of Phosphate buffer (pH 6.0 adjusted with 1M NaOH) and Acetonitrile in proportion of 60:40% v/v at a flow rate of 1.0 ml/min was used as a mobile phase. The injection volume was 20µl.

Preparation of mobile phase

[0.03M KH2PO4 pH6.0 adjusted with 1 N NaOH: ACN (60:40%v/v)]

Accurately weighed 4.08gm of Potassium dihydrogen phosphate was dissolved in 50 ml of water and dilute in a 1000 ml volumetric flask. Volume of resulted solution was made up to 1000 ml with water. pH of this buffer was adjusted to pH 6.0 with addition of 1M NaOH. Above solution filtered with vacuum filter using filter membrane. 40 ml of buffer and 60 ml Acetonitrile were mixed and solution was sonicated for degassing.

Diluents: Water: Acetonitrile (60:40) Preparation of Standard Stock Solution

(a) Standard Stock Solution of Bimatoprost (100 µg/ml**)**

Accurately weighed Bimatoprost (10mg) was transferred into 100 ml volumetric flask and diluted up to mark with diluents to give a stock solution (100µg/ml) of Bimatoprost.

(b) Standard Stock Solution of Timolol Maleate(500 µg/ml)

Accurately weighed Timolol Maleate (50 mg) was transferred into 100 ml volumetric flask, dissolved and diluted up to mark with diluents to give a stock solution (500µg/ml) of Timolol Maleate.

(c) Working Standard Solution of Bimatoprost & Timolol Maleate

Pipette out (1ml) from Stock solution of Bimatoprost and Pipette out (1ml) from Stock solution of Timolol Maleate than transferred in 10 ml volumetric flask and diluted up to mark with diluents to obtain working standard solution (10µg/ml) of Bimatoprost and (50µg/ml) of Timolol Maleate respectively.

Preparation of Sample Stock Solution

1ml Test Solution (which contain 0.3mg Bimatoprost & 5mg Timolol Maleate) & plus 0.7mg standard Bimatoprost were taken into 10ml volumetric flask dilute with diluents up to mark. Proper shake it and sonicate for sometimes. Pipette out 1ml from this solution in 10ml volumetric flask again dilute with diluents up to mark (Working test solution).

Method Validation

Validation of the analytical method is the process that established by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. The RP-HPLC method developed was validated according to International Conference on Harmonization[10] guidelines for validation of analytical procedures. The method was validated for the parameters in terms of specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantification (LOQ)

Specificity

Specificity is required to show that the procedure is unaffected by the presence of impurities or excipients. Specificity of an analytical method indicates that the analytical method is its able to measure accurately and specifically the analyte of interest without any interference from blank. Specificity was determined by the comparison of the chromatograms of blank, standard solution of individual & combined drug and sample solution.

Linearity & Range

For the linearity 100% each of working standard solution of Bimatoprost and Timolol Maleate were injected at Concentration 5-15 µg/ml and 25-75 µg/ml respectively and the results obtained are tabulated as follows in the table 2 and calibration curves are shown in figure 5 & 6. The results show an excellent correlation exists between mean peak area and concentrations level of drugs within the concentration range. The correlation coefficient for Bimatoprost and Timolol Maleate were found to be 0.999, and 0.999 respectively which is well within the acceptance criteria limits. The linearity study report is tabulated in table 3

Accuracy (%Recovery)

Accuracy was determined by calculating recovery of Bimatoprost and Timolol Maleate by the standard addition method. Known amounts of standard solutions of Bimatoprost (2.5, 5 and 7.5µg/ml) and Timolol Maleate (12.5, 25 and 37.5µg/ml) were added to a pre quantified test solutions of Bimatoprost (5µg/ml) and Timolol Maleate (25µg/ml). Each solution was injected in triplicate and the recovery was calculated by measuring peak areas. Results obtained are shown in table 4.

Precision

The method was validated in terms of intra-day and inter-day precision. The solution containing 10µg/ml of Bimatoprost and 50µg/ml of Timolol Maleate were injected six times for repeatability study. Inter-day and Intra-day study was performed by injecting 5, 10 and 15µg/ml of Bimatoprost and 25, 50 and 75µg/ml of Timolol Maleate solutions three times for each aliquots. The %RSD for precision study was found < 2% as shown in table 5, 6 & 7.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations as per International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$ $LOQ = 10 \times \sigma/S$

Where σ = the standard deviation of the response and S = Slope of calibration curve. The results are shown below table 8.

Robustness

The robustness study was performed to evaluate the influence of small but deliberate variation in the chromatographic condition. The robustness was checked by making three small changes. The mobile phase ration was changed by ± 0.2 ml, Flow rate changed by± 0.02 ml/min and pH changed by 0.1 After each changes sample solution was injected and system suitability parameters were observed. The results are shown in table 9**.**

RESULT AND DISCUSSION

Method Development

RP-HPLC method was developed as according to required limits of some parameters like number of theoretical plates, tailing factor, run time, resolution factor and cost effectiveness. This system suitability part is integral part of method which ensuring that adequate

performance of chromatographic system.

Table 1: Summary of System Suitability Parameters.

Parameters	Required Limits	Bimatoprost	Timolol Maleate
Retention time (min)	%RSD < 2%	6.357±0.080	2.203±0.022
Number of theoretical plates (N)	Not less than 2000	3652±2.00	3857±15.333
Tailing Factor (T)	Not more than 2	1.628	1.266

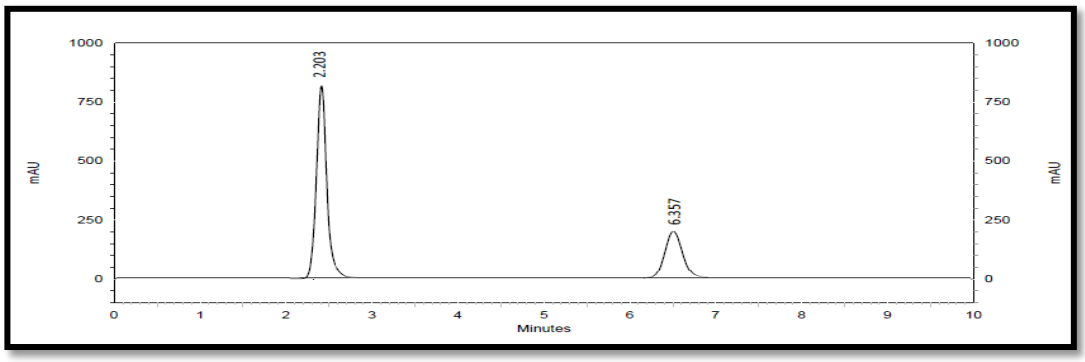

Figure 3: A typical RP-HPLC chromatogram of Bimatoprost (10µg/ml) and Timolol Maleate (50µg/ml) with corresponding retention time.

Method Validation Linearity

Table 2: Data for Linearity study of Bimatoprost and Timolol Maleate.

Sr. No.	Bimatoprost			
	Conc. (µg/ml)	Average Area (n=3)	SD	%RSD
1	5	1383.368	20.574	1.487
2	7.5	2038.131	14.906	0.731
3	10	2771.160	16.341	0.590
4	12.5	3487.847	8.346	0.239
5	15	4117.189	37.180	0.903
	Timolol Maleate			
Sr. No.	**Conc. (µg/ml)**	**Average Area (n=3)**	**SD**	**%RSD**
1	25	1645.433	5.983	0.363
2	37.5	2421.478	12.059	0.498
3	50	3287.821	16.364	0.498
4	62.5	4165.459	18.909	0.456
5	75	4877.619	14.737	0.301

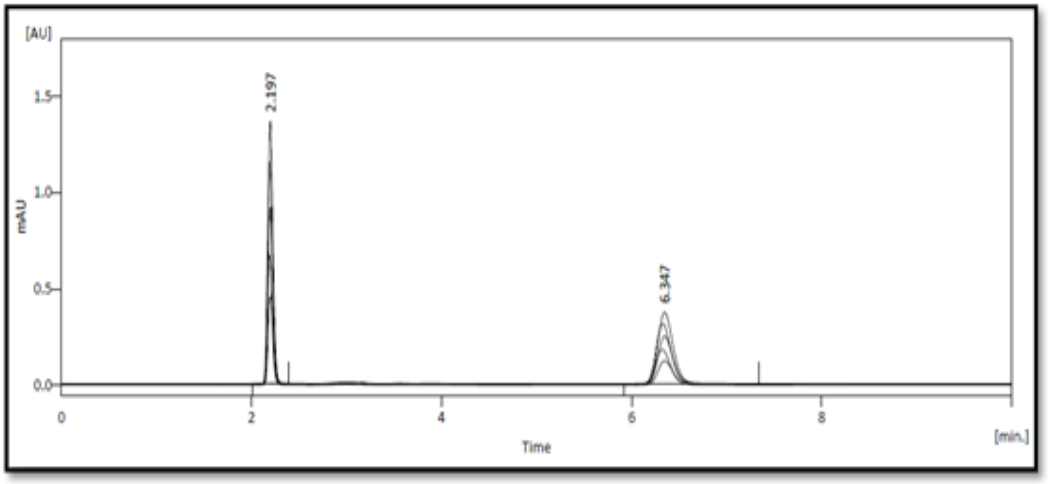

Figure 4: Overlain Chromatogram of Bimatoprost and Timolol Maleate.

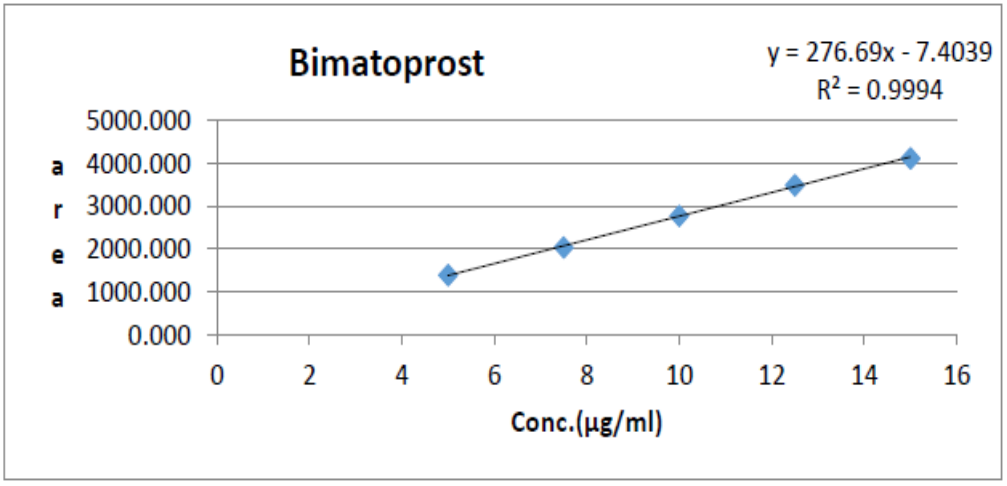

Figure 5: Calibration Curve of Bimatoprost.

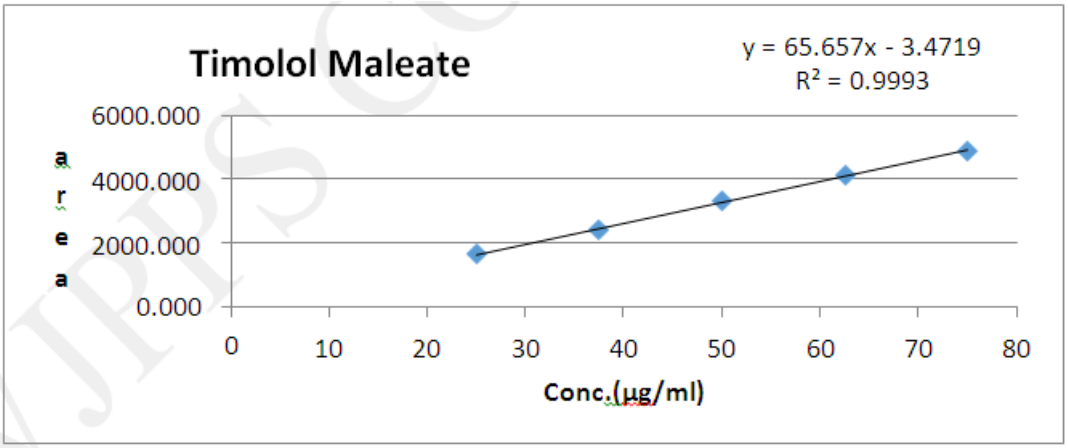

Figure 6: Calibration Curve of Timolol Maleate.

Table 3 Linearity Study Report.

Sr. No.	Drug	Regression Equation	Correlation Coefficient (R^2)	Slope (m)	Intercept (c)
1.	Bimatoprost	y=276.6x-7.403	0.999	276.6	7.403
2.	Timolol Maleate	y=65.65x-3.471	0.999	65.650	3.471

Accuracy

Table 4: Accuracy data of Bimatoprost and Timolol Maleate.

Drug	Sample Level %	Conc.(µg/ml)			Amt Recovered (n=3)	%Recovery
		Sample Amt	Amt Added	Total		
Bimatoprost	50	5	2.5	7.5	2.491	99.667 ± 0.187
	100	5	5.0	10	4.997	99.954 ± 0.792
	150	5	7.5	12.5	7.596	101.292 ± 0.974
Timolol Maleate	50	25	12.5	37.5	12.533	100.266 ± 1.104
	100	25	25	50	25.027	100.108 ± 0.685
	150	25	37.5	62.5	38.161	101.763 ± 0.951

Precision

Table 5: Intraday & Interday Precision Study of Bimatoprost.

Drug	Conc. (µg/ml)	Intraday Precision			Interday Precision		
		Mean (n=3)	SD	%RSD	Mean (n=3)	SD	%RSD
Bimatoprost	5	1366.871	19.3127	1.4129	1410.079	5.6638	0.4016
	10	2785.604	4.2225	0.1515	2715.515	35.1045	1.2927
	15	4119.471	20.4420	0.4962	4133.946	38.2766	0.9259

Table 6: Intraday & Interday Precision Study of Timolol Maleate.

Drug	Conc. (µg/ml)	Intraday Precision			Interday Precision		
		Mean (n=3)	SD	%RSD	Mean (n=3)	SD	%RSD
Timolol Maleate	25	1630.755	5.6564	0.3468	1670.686	6.7248	0.4025
	50	3304.142	9.8930	0.2994	3224.757	27.5654	0.8548
	75	4900.387	36.8089	0.7511	4901.388	44.4259	0.9063

Table 7: Repeatability Study of Bimatoprost & Timolol Maleate.

Bimatoprost		Timolol Maleate	
Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
10	2778.988	50	3291.007
10	2792.850	50	3307.448
10	2745.852	50	3281.230
10	2801.085	50	3317.117
10	2829.145	50	3350.285
10	2834.791	50	3356.959
Average	**2797.119**	**Average**	**3317.841**
SD	**32.97222**	**SD**	**30.13679**
% RSD	**1.178792**	**% RSD**	**0.9083255**

LOD & LOQ

Table 8: Result of LOD & LOQ.

Sr. No.	Drug	LOD	LOQ
1	Bimatoprost	0.382 µg/ml	1.157 µg/ml
2	Timolol Maleate	1.929 µg/ml	5.845 µg/ml

Robustness

Table 9: Robustness Study of Bimatoprost & Timolol Maleate.

Parameters	Change Level	Area (n=3)	
		Bimatoprost	Timolol Maleate
Mobile Phase Composition Buffer: ACN (± 2.0 ml)	62:38	2734.852	3235.258
	60:40	2792.850	3307.448
	58:42	2835.638	3317.17
	Mean ±SD	2787.78 ± 50.883	3286.722 ± 44.849
	%RSD	1.814	1.364
pH (±0.1)	6.1	2790.978	3314.139
	6.0	2778.988	3317.17
	5.9	2733.824	3237.392
	Mean ±SD	2767.93 ± 30.138	3289.549 ± 45.19
	%RSD	1.088	1.374
Flow Rate (±0.02 ml/min)	1.02 ml/min	2705.31	3203.548
	1.0 ml/min	2778.988	3317.117
	0.98 ml/min	2735.638	3264.529
	Mean ±SD	2739.979 ± 37.030	3261.731 ± 56.836
	%RSD	1.351	1.742

Table 10: % Assay of Bimatoprost & Timolol Maleate.

Sr. No.	Drug	Label Claim (mg)	Conc. Taken for % Assay (µg/ml)	Mean Peak Area (n=3)	Mean %Assay ± SD
1	Timolol Maleate	5	50	3168.783	96.284 ± 0.5744
2	Bimatoprost	0.3	10	2664.569	95.883 ± 0.924

CONCLUSION

Simple, accurate, rapid and precise RP-HPLC method was developed and validated for simultaneous estimation of both these drugs. Developed method was validated as per ICH guidelines those exhibited excellent specificity, linearity, precision, accuracy, Robustness. The results indicate that method is equally sensitive, reliable and can routinely apply for simultaneous estimation of Bimatoprost and Timolol Maleate in its Pharmaceutical formulation.

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