

# A Stability Indicating Reverse Phase-HPLC Method Development and Validation for the Estimation of Bimatoprost 0.3% & Timolol 0.5% Pharmaceutical Ophthalmic Dosage Form

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## Abstract

The research was carried out to establish a new reverse phase-HPLC stability indicating method for quantifying Bimatoprost & Timolol in ophthalmic solution. The experiment of Bimatoprost & Timolol in ophthalmic solution method development was determined on Waters HPLC instrument using a UV Detector. The separation was done by using L11, Zorbex SB phenyl (4.6 mm × 250 mm internal diameter) 5 μm analytical column, containing mobile phase of Phosphate buffer (0.02 M), methanol, and acetonitrile [50:30:20 % v/v]. The method was run at 1 ml·min<sup>-1</sup> at 210 nm for Bimatoprost and 295 nm for Timolol for detection. The drug was eluted at 10.81 min for Bimatoprost and 3.77 min for Timolol. After developing the method, it was assured for the intended use by validation, which was done according to ICH Q2B guidelines. The analytical parameters checked were Specificity/Selectivity, linearity, Range, accuracy, ruggedness, and robustness. It was observed that the response of the detector was linear in the range of 6 - 18 μg/ml with a correlation coefficient of 0.999. The results of all the parameters were found to be within the acceptance criteria. The stability-indicating assay method was established by using the samples generated by the forced degradation process. The forced degradation was carried out by subjecting the drug to acid, alkali, thermal, oxidative, and photolytic degradation, and the results showed that the degradation products were successfully separated from the drug. Hence, this can be applied perfectly later for the quantitative analysis of Bimatoprost 0.3% + Timolol 0.5% Ophthalmic Solution drugs for pharmaceutical use. Currently, there is no official method for Bimatoprost & Timolol combination products in USP or BP. Available research work related to single Bimatoprost & Timolol combination products in USP or BP.

toprost or Timolol products was not suitable for testing Bimatoprost and Timolol combination drugs. Additionally, there is no stability-indicating method to test Bimatoprost & Timolol combination products which insist us to do research and develop a new reverse phase-HPLC indicating method which will be faster and more accurate.

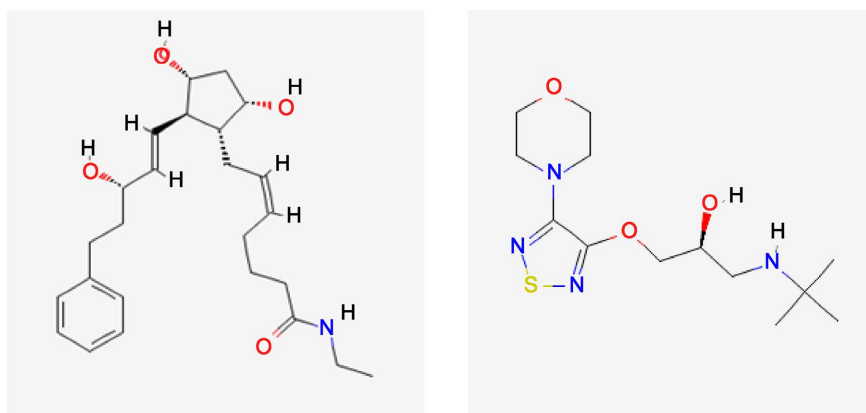
## Keywords

Bimatoprost, Timolol, Reverse-Phase High Performance Liquid Chromatography, Method Development, Validation, Stability Indicating

## 1. Introduction

Bimatoprost chemically, (*Z*)-7-[(1*R*, 2*R*, 3*R*, 5*S*)-3,5-dihydroxy-2-[(*E*,3*S*)-3-hydroxy-5-phenylpent-1-enyl]cyclopentyl]-*N*-ethylhept-5-enamide is a synthetic prostamide and structural prostaglandin analogue [1]. Bimatoprost mimics the effects of the endogenous prostamides and reduces intraocular pressure by increasing outflow of aqueous humor through both the pressure-sensitive outflow pathway (the trabecular meshwork), and the pressure-insensitive outflow pathway (the uveoscleral routes). It is not clear whether Bimatoprost lowers intraocular pressure by stimulating F-Prostanoid receptors or by acting on specific prostamide receptors.

Timololchemically, (2*S*)-1-(*tert*-butylamino)-3-[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]propan-2-ol is a nonselective beta-adrenergic receptor blocker that is widely used for the therapy of hypertension, angina pectoris and prevention of vascular headaches [2]. Timolol is a beta-adrenergic antagonist; both the hemihydrate and the maleate salt are used in the management of glaucoma, hypertension, angina pectoris and myocardial infarction, and for the prevention of migraine. Timolol is a nonselective beta-adrenergic antagonist given in an eye drop solution to reduce intraocular pressure, or pressure in the eyes.



The complete information with data supplemented from the literature that

there are only a few methods like HPLC [3]-[9] techniques but these methods are not for Bimatoprost + Timolol combination and the method is not stability indicating. Therefore, there was no simple method developed to date, which is accurate and simple for the analysis of Bimatoprost + Timolol Ophthalmic Solution. Hence, it felt necessary to establish a new, easier, cost-effective, precise, accurate, and specific stability-indicating analytical method that can be easily applicable to routine drug performance evaluations.

## 2. Materials and Methods

### 2.1. Materials

The investigated sample Bimatoprost [Fleming Laboratories Ltd.] and Timolol [Enaltec Labs] were procured from local market, Dhaka. The Bimatoprost & Timolol Ophthalmic Eye Drops was collected from local Pharmaceutical Company in Dhaka. Acetonitrile [Merck], Methanol [Merck], Ortho Phosphoric Acid [Merck], Sodium Dihydrogen Phosphate [Merck], were of HPLC grade and Purified water was generated using HPLC Mille-Q.

### 2.2. Equipment

Waters HPLC system equipped with UV detector and L11, Zorbex SB phenyl (4.6 mm × 250 mm internal diameter) 5 µm analytical column was used for quantitation of Bimatoprost and Timolol. The processing was done with complete data obtained from Empower-3 software. The sample's solubility in the mobile phase was enhanced using Ultra-sonic Bath (DU22 model) Copoly Scientific UK. All the samples prepared were weighted using Micro Balance (EP205) by Mettler Toledo.

## 3. Methodology

### 3.1. Chromatographic Conditions

The reverse-phase chromatography was performed on Waters HPLC using UV detection. The separation was done by using L11, Zorbex SB phenyl (4.6 mm × 250 mm internal diameter) 5 µm analytical column containing Phosphate buffer (0.02 M), methanol, and acetonitrile [50:30:20 % v/v] mobile phase. The method was run at 1 ml·min<sup>-1</sup> at 210 nm for Bimatoprost and 295 nm for Timolol UV detection. The elution time for the Bimatoprost drug was 10.81 min and Timolol for 3.77 min.

### 3.2. Preparation of Bimatoprost and Timolol Stock Working Standard Solutions

#### 3.2.1. Preparation of Stock Standard Solution of Bimatoprost

Weigh accurately and transfer about 24 mg of Bimatoprost working standard into a 100 ml clean and dried volumetric flask. Add about 65 ml of mobile phase, shake and sonicate to dissolve. Cool the solution to room temperature, make volume up to 100 ml with mobile phase, and mix well. The concentration of Bi-

matoprost will be 0.24 mg/ml.

### 3.2.2. Preparation of Stock Standard Solution of Timolol

Weigh accurately and transfer about 27.3 mg of Timolol Maleate working standard into a 100 ml clean and dried volumetric flask. Pipette 5 ml of the stock standard solution of Bimatoprost into the same volumetric flask. Add about 60 ml of mobile phase, shake and sonicate to dissolve. Cool the solution to room temperature, make volume up to 100 ml with mobile phase, and mix well. The concentration of Bimatoprost will be 0.012 mg/ml and the Concentration of Timolol Maleate will be 0.273 mg/ml.

### 3.3. Preparation of Sample Solution

Pipette 4 ml of the Bimatoprost 0.03% & Timolol 0.5% eye drop into 100 ml clean and dried volumetric flask. Make volume up to 100 ml with mobile phase and mix well. Concentration of Bimatoprost will be 0.012 mg/ml and Concentration of Timolol Maleate will be 0.273 mg/ml.

## 4. Method Validation

As per ICH guidelines, the checked validation parameters were Specificity, linearity, Accuracy, Precision, Range, Ruggedness, and Robustness was checked [10] [11].

### 4.1. System Suitability

For evaluating the suitability of the HPLC system and procedure, the Bimatoprost & Timolol standard solution of about 10  $\mu$ l was introduced into the HPLC system Six-time and recorded the chromatogram. The acceptance criteria of RSD of six injections were not more than 2.0.

### 4.2. Specificity

Specificity is the ability of the method to accurately measure the analyte responses in presence of all formulation ingredients. A study was designed to demonstrate the effective separation of Bimatoprost and Timolol. In addition, the study was intended to ensure the effective separation of degradation peaks of formulation ingredients at the retention time of Bimatoprost and Timolol.

### 4.3. Linearity

The Linear response of Bimatoprost and Timolol was established by plotting a graph of concentration versus area and determining the correlation coefficient. Standard solutions of Bimatoprost and Timolol to about 50%, 80%, 100%, 120%, and 150% of the target concentration were prepared single time and injected each solution three times. A graph was plotted by placing concentration (mg/ml) on X-axis versus area on Y-axis. The correlation coefficient was calculated for principal peaks. From the results slope, intercept, and correlation coefficient (r)

of the least regression line was calculated.

#### **4.4. Accuracy**

The test sample solutions were prepared as per spiked sample preparation at about 50%, 100% & 150% of the target concentration. Each sample solution was prepared three times at each spiked level and injected into the HPLC system a single time. The % recovery for the assay was calculated.

#### **4.5. Precision**

##### **4.5.1. Intra-Day Assay Precision**

To evaluate the intra-day precision, 10 µg/ml concentration solution was injected for five times under unchanged conditions within a short period. The peak areas for the five replicate injections were collected and calculated the % Relative standard deviation.

##### **Procedure: (Day 01, Repeatability)**

The precision of the test method was evaluated by six spiked samples. The system suitability parameters were evaluated. A standard solution was injected into the HPLC system and chromatograms were recorded.

##### **4.5.2. Intermediate Precision**

To Evaluate the Intermediate precision, the sample was analyzed on different days under unchanged conditions.

##### **Procedure: (Day 02, Intermediate precision)**

The Intermediate precision of the test method was evaluated by six samples.

#### **4.6. Range**

From the linearity, accuracy, and precision of the method, the range was determined over a range of concentrations.

#### **4.7. Ruggedness**

Robustness was evaluated by slightly changing the chromatographic conditions, which includes percent organic solvent and flow rate. System to System, Analyst to Analyst, and Column to column variation was considered for the ruggedness study.

#### **4.8. Robustness**

Standard solution stability, Sample solution stability, Mobile phase stability, Flow rate variation, Column oven temperature Variation, Flush volume variation (Filter), Variation in pH of the buffer of the mobile phase, and Mobile phase composition variation were considered during the robustness study.

##### **4.8.1. Solution Stability**

##### **1) Standard Solution Stability**

Standard preparation was injected for precision study at different time inter-

vals such as initial and after 72 hours. The solution was stored at two different conditions *i.e.* at room temperature and 2°C - 8°C temperature during the course of the validation experiment and the peak area for Bimatoprost & Timolol was recorded and potency was calculated for the standard and compared with the initial result (100%). Each time a freshly prepared standard solution was injected and re-analyzed.

### **2) Sample Solution Stability**

Sample preparation used for the precision study was injected at different time intervals such as initial and after 72 hours. The solution was stored at two different conditions *i.e.* at room temperature and 2°C - 8°C temperature during the course of the validation experiment and the peak area was recorded for Bimatoprost & Timolol to calculate the recovery of Bimatoprost & Timolol in comparison with the initial result. Each time a freshly prepared standard solution was injected and re-analyzed.

### **3) Mobile Phase Stability**

System suitability parameters were monitored for the standard solution at different time intervals during the course of the validation experiment using the mobile phase that is prepared at least 72 hours before. The standard solution was injected six times and six sample preparations were used for the precision study. System suitability was monitored and the % assay of six individual samples was calculated.

### **4) Variation in Flow Rate**

Six standard solutions and six sample solutions were injected to use for precision study at two different flow rates (0.9 ml/minute and 1.1 ml/minute). System suitability was monitored and the % assay of six individual samples was calculated.

### **5) Column Oven Temperature Variation**

Six standard solutions and six sample solutions were injected to use for precision study at two different column temperatures (35°C & 37°C). System suitability was monitored and the % assay of six individual samples was calculated.

### **6) Variation in Flush volume (Filter)**

Discard the volume of sample solution (5 ml, 10 ml, 15 ml, and 20 ml), and the assay of Bimatoprost & Timolol was calculated in each time to check the effectiveness of discard volume during filtration. Samples without filters were also injected.

### **7) Effect of Mobile Phase Composition (Buffer and organic Phase) Variation**

The standard solution was injected six times and six samples were used for precision study by using two different mobile phase composition (buffer: organic phase = 48:52 & 52:48). System suitability was monitored and the % assay of six individual samples was calculated.

#### **4.8.2. Effect of Variation in pH of Buffer of Mobile Phase**

The standard solution was injected six times and six samples were used for

preparations used for precision study by using two different pH of the buffer of mobile phase (pH 2.7 and 2.9). System suitability was monitored and the % assay of six individual samples was calculated.

## **5. Forced/Stress Degradation Studies**

The specificity of the method was determined by subjecting the active drug to force/stress conditions such as acid Hydrolysis, Base Hydrolysis, Peroxide Oxidation, Thermal degradation, and Photolytic Degradation. In addition, a control solution was analyzed to determine the degradation.

The samples and standards under stress condition were monitored in order to demonstrate detectable interference due to degradants or any other compounds formed. In order to see the reactivity of inactive placebo with Bimatoprost and Timolol under stress conditions, the placebo mixture (without the actives) was also treated under the same conditions. Chromatograms were recorded and the purity of the peak was determined by calculating the % of degraded amount and % of the active amount.

### **5.1. Acid Degradation**

Concentrated Hydrochloric acid solution was added to the sample and standard solution. The solution was heated in the water bath at 85°C which was obtain approximately 10% to 20% degradation of assay value, wherever practically possible. This solution neutralized with the same volume and same strength alkali solvent and diluted to 100 ml with the mobile phase.

### **5.2. Basic Degradation**

Concentrated Sodium Hydroxide solution added to the sample and standard solution. The solution was heated in the water bath at 85°C which was obtain approximately 10% to 20% degradation of assay value, wherever practically possible. This solution neutralized before dilution with the same volume and same strength acid and diluted to 100 ml with the mobile phase.

### **5.3. Oxidative Degradation**

Concentrated Hydrogen Peroxide solutions added to the sample and standard solution. The solution heated in the water bath at 85°C, which will obtain approximately 10% to 20% degradation of assay value, wherever practically possible. This solution diluted to 100 ml with the mobile phase.

### **5.4. Thermal Degradation**

The solution in the water bath was heated at 85°C, to obtain approximately 10% to 20% degradation of assay value, wherever practically possible, and diluted to 100 ml with the mobile phase.

### **5.5. Photo Degradation**

The sample exposed to direct sunlight to obtain approximately 10% to 20% de-

gradation of assay value, wherever practically possible, and diluted to 100 ml with the mobile phase.

### 5.6. Control

Without treatment.

## 6. Results and Discussions

### 6.1. Method Development

For this method development, various ratios and combinations of mobile phases, different stationary phases, and flow rates were tried to elute the drug with good peak parameters and to provide good performance in the assay. Finally, the best separation was achieved on 4.6 mm × 250 mm, 5 μm, L11, Zorbex SB phenyl column comprising mobile phase of pH 2.8 phosphate buffer, methanol, and acetonitrile at a ratio of (50:30:20). The method was run at a flow rate of 1.0 ml·min<sup>-1</sup> and the eluent was detected at 10.81 min for Bimatoprost and 3.77 min for Timolol by UV detector at 210 nm for Bimatoprost and 295 nm for Timolol. The chromatogram showed the peak with good shape, and more theoretical plates and the tailing factor was also found to be within the limits. All the method development trials with various mobile phase compositions, column and flow rates results were shown in **Table 1**.

The standard chromatogram of Bimatoprost and Timolol drug was presented in **Figure 1(a)** & **Figure 1(b)**.

### 6.2. Analytical Method Validation

#### 6.2.1. System Suitability

The theoretical plate number, peak asymmetry, and percentage relative standard deviation obtained were within the acceptance criteria and demonstrated that the method can generate accurate and precise results. The results were presented in **Table 2**.

#### 6.2.2. Specificity

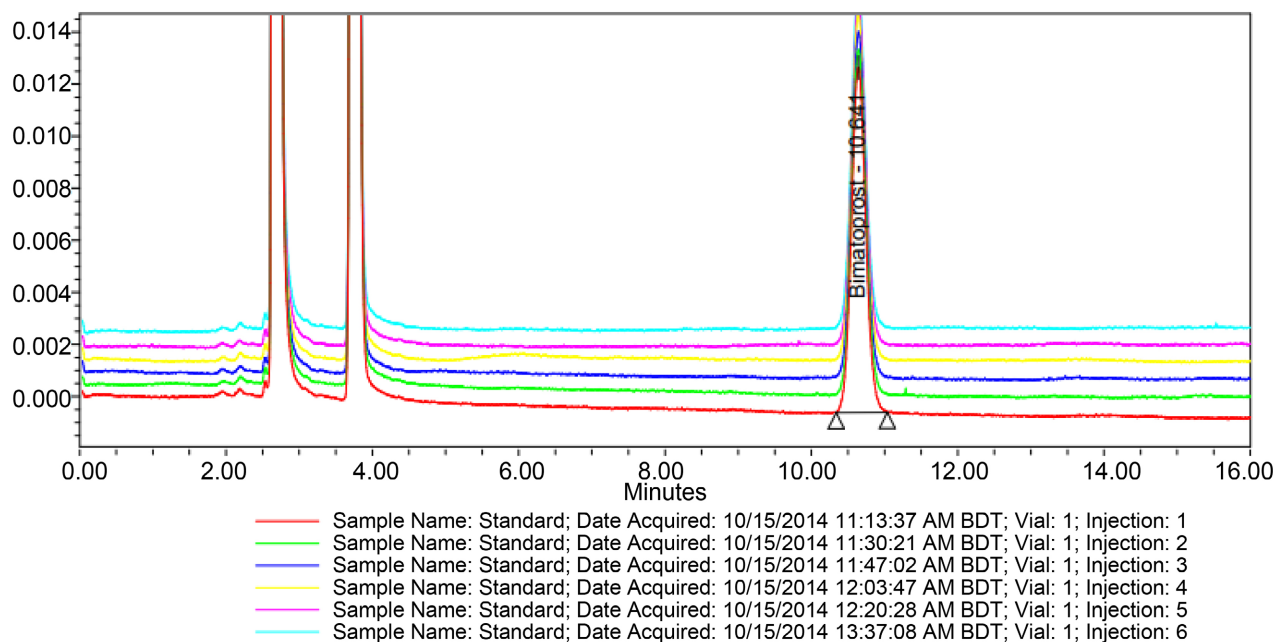
It was found that the peak of Bimatoprost and Timolol was effectively separated in presence of all formulation ingredients. Acceptance criteria were “there should be no interfering peak in the chromatogram obtained from diluent or placebo solution at the retention time corresponding to Bimatoprost and Timolol”, results should be comparable with respect to retention time and purity angle must be less than purity threshold for Bimatoprost and Timolol. The measured results were found satisfactory which is provided in **Table 3(a)** & **Table 3(b)**.

#### 6.2.3. Linearity.

Linear response of Bimatoprost and Timolol was established by plotting a graph to concentration versus area and determining the correlation coefficient. Standard solutions of Bimatoprost and Timolol to about 50%, 80%, 100%, 120%, and

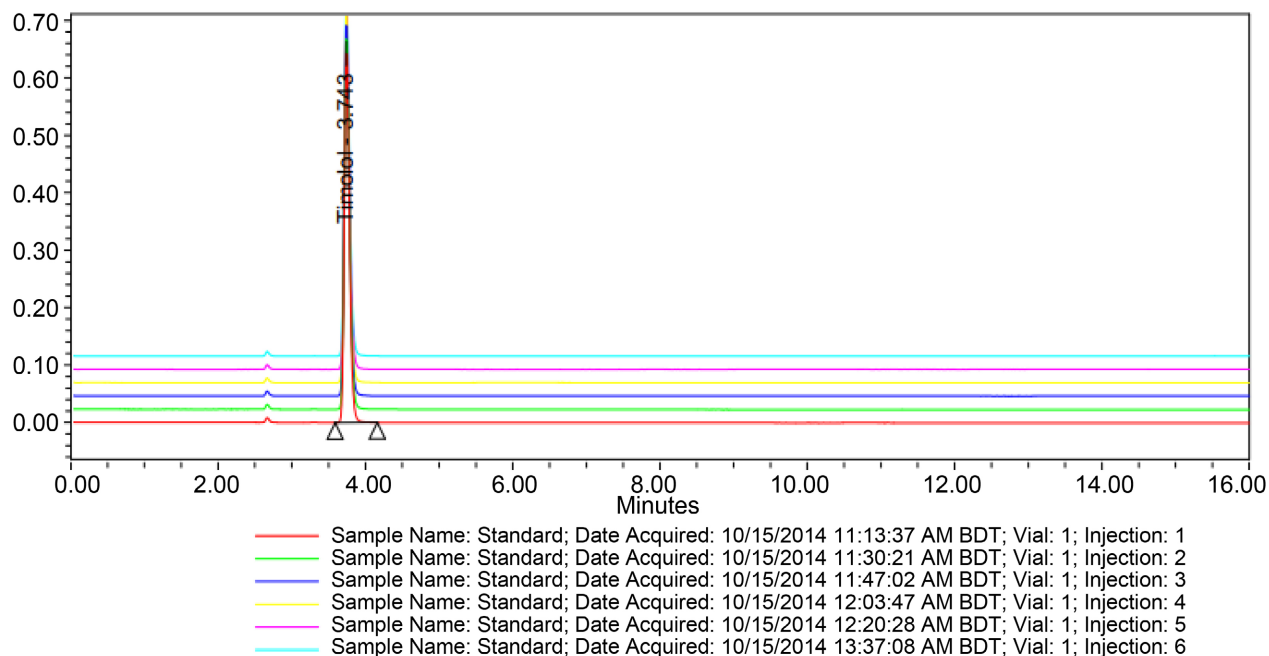


## Peak Summary Report



(a)

## Peak Summary Report



(b)

**Figure 1.** (a): Optimized chromatogram standard (Bimatoprost). (b): Optimized chromatogram standard (Timolol).

150% of the target concentration were prepared single time and injected each solution three times. A graph was plotted by placing concentration (mg/ml) on X-axis versus the area on Y-axis. The correlation coefficient was calculated for

**Table 1.** Method development trials and observation.

Chromatographic Conditions					
S. No.	Analytical Column	Mobile phase composition	Flow rate	Retention time (min)	Result
1)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	pH 2.8 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	0.9 ml/min	11.99 min & 4.18 min	Improper peak separation and more RT
2)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	pH 2.8 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.0 ml/min	10.81 min & 3.77 min	More RT but pack separation, theoretical plate and trailing factor was good.
3)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	pH 2.8 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.1 ml/min	9.84 min & 3.43 min	Improper peak separation and more RT
4)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	pH 2.7 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.0 ml/min	10.74 min & 3.76 min	Improper peak separation and more RT
5)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	pH 2.9 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.0 ml/min	10.36 min & 3.74 min	Improper peak separation and more RT
6)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	48:52 Mobile Phase Ratio	1.0 ml/min	8.78 min & 3.58 min	Improper peak separation, more tailing and less plate count
7)	Zorbex SB phenyl, L11 (4.6 mm × 250 mm) 5 μm	52:48 Mobile Phase Ratio	1.0 ml/min	12.13 min & 3.89 min	Improper peak separation and more RT
8)	Phenomenex C18 ODS (4.6 × 150 mm) 5 μm	pH 2.8 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.0 ml/min	7.8 min & 3.42 min	Improper peak separation, more tailing and less plate count
9)	Inertsil C18 (15 cm × 0.46 cm) 5 μm	pH 2.8 phosphate buffer, methanol and acetonitrile at a ratio of (50:30:20)	1.0 ml/min	14.2 min & 4.9 min	Improper peak separation and more RT

**Table 2.** System suitability data.

S. No.	Parameters	Bimatoprost	Timolol
1	Retention Time	10.81	3.77
2	Plate count	<b>14,260</b>	<b>13,584</b>
3	Tailing Factor	<b>1.07</b>	<b>1.28</b>
4	Peak Area (AUC)	<b>183,932</b>	<b>3,228,378</b>

**Table 3.** Bimatoprost & timolol peak identification and peak purity with placebo interference. (a) Peak identification and peak purity. (b) Placebo Interference.

(a)				
Peak name	Sample type	Retention time	Purity angle	Purity threshold
<b>Bimatoprost</b>	Standard	10.81	0.22	<b>0.48</b>
	Sample	10.81	0.23	<b>0.49</b>
<b>Timolol</b>	Standard	3.77	0.12	<b>0.25</b>
	<b>Sample</b>	<b>3.77</b>	<b>0.11</b>	<b>0.24</b>

(b)		
Sample No	% Interference with Bimatoprost	% Interference with Timolol
1	Nil	Nil

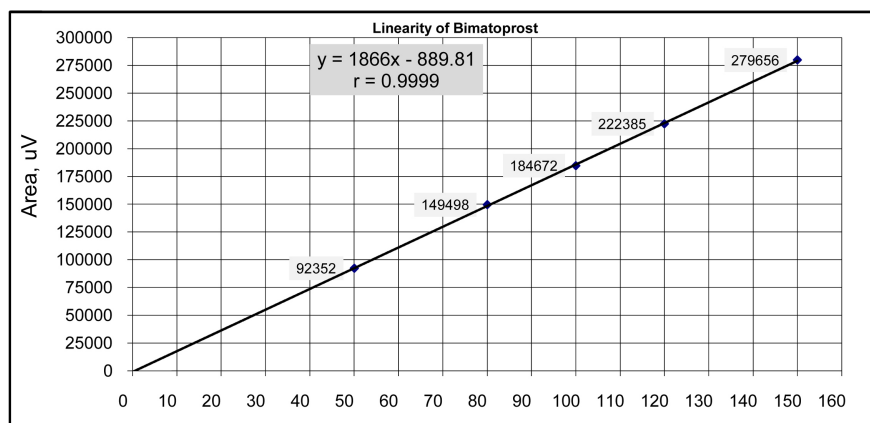
principal peaks. Acceptance criteria for Bimatoprost and Timolol were not less than 0.999 and the % limit of the Y-intercept should be within  $\pm 2$  of the corresponding Y-co-ordinate of the working level. From the results slope, intercept, and correlation coefficient (r) of the least regression line was calculated and the results were within the limit. These results are summarized in **Table 4** and **Figure 2(a)** & **Figure 2(b)**.

#### 6.2.4. Accuracy

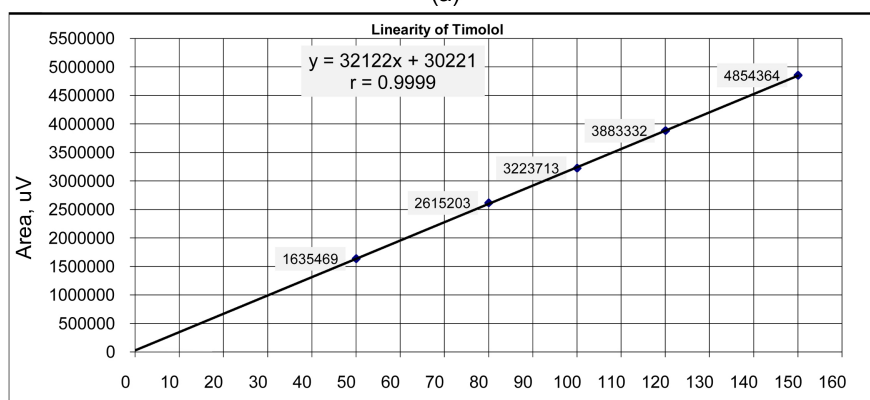
The test sample solutions were prepared as per spiked sample preparation at about 50%, 100% & 150% of the target concentration. Each sample solution was prepared three times at each spiked level and injected into the HPLC system a single time. Acceptance criteria were “percentage of recovery should be less than 98.0 and should not be more than 102.0 and RSD of nine determination should not be more than 2.0”. The % recovery for the assay was calculated. The mean recovery of Bimatoprost and Timolol actual versus theoretical concentration was calculated to be  $99.41\% \pm 0.49\%$  for Bimatoprost and  $100.30\% \pm 0.29\%$  for Timolol. Hence the HPLC method can be considered to be accurate for Bimatoprost and Timolol determinations. The measured results are provided in **Table 5**.

#### 6.2.5. Precision

The precision of the test method was evaluated by six spiked samples and the system suitability parameters were evaluated and found within the limits. The % RSD obtained was within the limits indicating the methods precision. Acceptance criteria were “% RSD of Bimatoprost and Timolol retention time should be not more than 2.0, % RSD of Bimatoprost and Timolol area should not be more than 2.0, The tailing factor of the Bimatoprost and Timolol peak is not more than 2.0 and Theoretical plate count for the Bimatoprost and Timolol peak is not



(a)



(b)

**Figure 2.** (a) and (b): Linearity curve for Bimatoprost and Timolol.**Table 4.** Linearity of Bimatoprost and Timolol.

Linearity of Bimatoprost		Linearity of Timolol	
Concentration, (mg/ml)	Area of Bimatoprost peak	Concentration, (mg/ml)	Area of Timolol peak
0.0060	92,352	0.1365	1,635,469
0.0096	149,498	0.2184	2,615,203
0.0120	184,672	0.2730	3,223,713
0.0144	222,385	0.3276	3,883,332
0.0180	279,656	0.4095	4,854,364
Slope	1866	Slope	32,122
Y-intercept	889.81	Y-intercept	30,221
Median (Area)	184,672	Median (Area)	3,223,713
% of Y intercept	0.48	% of Y intercept	0.94
Correlation coefficient, r	0.9999	Correlation coefficient, r	0.9999

**Table 5.** Accuracy of Bimatoprost and Timolol. (a) Accuracy (Bimatoprost). (b) Accuracy (Timolol).

(a)					
Sample No	Spiked level (%)	Amount of Bimatoprost added in $\mu\text{g}$	Amount of Bimatoprost found in mg	% of Recovery	Mean & % RSD
1	50	601.60	592.22	98.44	
2	50	600.00	594.96	99.16	98.92% & 0.42%
3	50	600.80	595.75	99.16	
4	100	1204.80	1210.46	100.47	
5	100	1205.60	1192.34	98.90	99.75% & 0.79%
6	100	1201.60	1200.16	99.88	
7	150	1832.00	1817.34	99.20	
8	150	1816.00	1827.80	100.65	99.90% & 0.73%
9	150	1824.00	1821.26	99.85	
Grand average (%)					99.52%
RSD of nine determinations					0.74%
95% confidence interval					99.52 $\pm$ 0.48
(b)					
Sample No	Spiked level (%)	Amount of Bimatoprost added in $\mu\text{g}$	Amount of Bimatoprost found in mg	% of Recovery	Mean & % RSD
1	50	13768.00	13887.78	100.87	
2	50	13728.00	13895.48	101.22	100.01% & 0.19%
3	50	13752.00	13879.89	100.93	
4	100	27304.00	27353.15	100.18	
5	100	27320.00	27333.66	100.05	100.11% & 0.07%
6	100	27320.00	27350.05	100.11	
7	150	40968.00	41201.52	100.57	
8	150	41000.00	41254.20	100.62	100.59% & 0.03%
9	150	41016.00	41253.89	100.58	
Grand average (%)					100.57%
RSD of nine determinations					0.40%
95% confidence interval					100.57 $\pm$ 0.26

less than 2000. Results of intraday precision and intermediate precision respectively are depicted in **Table 6** & **Table 7**.

### 6.2.6. Range of Assay Method

The linearity, precision, and accuracy of the HPLC assay method were determined over a range of concentrations.

**Table 6.** Precision (Day-01, Repeatability). (a) Sample precision (Assay of Bimatoprost). (b) Sample precision (Assay of Timolol).

(a)						
Sample Number	Amount of Bimatoprost added in $\mu\text{g}$	Amount of Bimatoprost found in $\mu\text{g}$	% of Assay	Average Result (%)	RSD (%)	95% confidence Interval
1	1200.00	1199.88	99.99			
2	1200.00	1202.28	100.19			
3	1200.00	1203.36	100.28			
4	1200.00	1206.72	100.56	100.16	0.30	100.16 $\pm$ 0.24
5	1200.00	1196.28	99.69			
6	1200.00	1203.24	100.27			
(b)						
Sample Number	Amount of Timolol added in $\mu\text{g}$	Amount of Timolol found in $\mu\text{g}$	% of Assay	Average Result (%)	RSD (%)	95% confidence Interval
1	27320.00	27380.10	100.22			
2	27320.00	27300.88	99.93			
3	27320.00	27311.80	99.97			
4	27320.00	27311.80	99.97	99.98	0.12	99.98 $\pm$ 0.10
5	27320.00	27295.41	99.91			
6	27320.00	27289.95	99.89			

The HPLC method was shown to be linear and had an intercept of zero over a concentration range of 0.0060 mg/ml to 0.0180 mg/ml for Bimatoprost and 0.10 mg/ml to 0.30 mg/ml for Timolol. This corresponded to a concentration range of 50% to 150% sample concentrations.

The precision of the HPLC system was determined at a concentration of 100%. It was determined by performing six preparations. The % RSD of recovery of Bimatoprost (0.30%) and Timolol (0.12%) exhibited that the system was precise at 100%.

The accuracy of the HPLC method was determined over the range of 0.0060 mg/ml to 0.0180 mg/ml for Bimatoprost and 0.10 mg/ml to 0.30 mg/ml for Timolol. This corresponds to a concentration range of 50% to 150% sample concentrations.

### 6.2.7. Raggedness

System-to-system variability study was conducted on two HPLC systems by using two different columns by using six different test preparations under similar conditions. The system suitability parameters were evaluated as per the test method on both the systems and found within limits. Results were presented in **Table 8**.

**Table 7.** Precision (Day-02, Repeatability). (a) Sample precision (Assay of Bimatoprost). (b) Sample precision (Assay of Timolol).

(a)						
Sample Number	Amount of Bimatoprost added in $\mu\text{g}$	Amount of Bimatoprost found in $\mu\text{g}$	% of Assay	Average Result (%)	RSD (%)	95% confidence Interval
1	1200.00	1205.88	100.49	100.36	0.40	100.36 $\pm$ 0.32
2	1200.00	1211.04	100.92			
3	1200.00	1202.28	100.19			
4	1200.00	1205.16	100.43			
5	1200.00	1204.92	100.41			
6	1200.00	1196.28	99.69			
(b)						
Sample Number	Amount of Timolol added in $\mu\text{g}$	Amount of Timolol found in $\mu\text{g}$	% of Assay	Average Result (%)	RSD (%)	95% confidence Interval
1	27320.00	27229.84	99.67	99.78	0.27	99.78 $\pm$ 0.22
2	27320.00	27284.48	99.87			
3	27320.00	27388.30	100.25			
4	27320.00	27218.92	99.63			
5	27320.00	27259.90	99.78			
6	27320.00	27177.94	99.48			

**Table 8.** Ruggedness-system to system variability.

HPLC System Configuration	HPLC System-1		HPLC System-2	
Column Information	Column-1		Column-2	
	Recovery of Bimatoprost & Timolol as % of labeled amount			
Sample No.	System-1		System-2	
	Column-1		Column-2	
	Bimatoprost	Timolol	Bimatoprost	Timolol
1	99.99	100.22	100.66	100.19
2	100.19	99.93	99.86	99.58
3	100.28	99.97	100.17	99.72
4	100.56	99.97	99.90	99.66
5	99.69	99.91	100.02	99.62
6	100.27	99.89	99.87	99.47
<b>Mean</b>	<b>100.16</b>	<b>99.98</b>	<b>100.08</b>	<b>99.71</b>
<b>RSD</b>	<b>0.30</b>	<b>0.12</b>	<b>0.31</b>	<b>0.25</b>

### 6.2.8. Robustness

Upon slight changes in the Solution column oven temperature variation, flow rate, pH of buffer solution, and percent organic solvent, the results confirmed the reliability of the method. Results were presented in **Tables 9-12**.

**Table 9.** Robustness-Column oven temperature. (a)-(d) Column oven set at 33°C (Bima-toprost).

(a)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	11.09	184281	1.09	13583	99.94	100.03 ± 0.10
02	11.09	184720	1.08	14165	100.18	
03	11.09	184328	1.06	13833	99.96	
04	11.09	184627	1.06	13640	100.13	
05	11.09	184143	1.07	13637	99.86	
06	11.09	184629	1.05	13617	100.13	
<b>Average</b>	<b>11.09</b>	<b>184455</b>	<b>1.07</b>	<b>13746</b>	<b>100.03</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.13</b>	<b>N/A</b>	<b>N/A</b>	<b>0.13</b>	
(b)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3,215,953	184,281	1.29	13,131	99.87	100.07 ± 0.13
02	3,230,673	184,720	1.29	13,261	100.33	
03	3,221,577	184,328	1.29	13,179	100.04	
04	3,218,987	184,627	1.28	13,330	99.96	
05	3,221,804	184,143	1.29	13,101	100.05	
06	3,225,487	184,629	1.29	13,115	100.17	
<b>Average</b>	<b>3,222,414</b>	<b>184,455</b>	<b>1.29</b>	<b>13,186</b>	<b>99.87</b>	
<b>% RSD</b>	<b>0.16</b>	<b>0.13</b>	<b>N/A</b>	<b>N/A</b>	<b>100.07</b>	
(c)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	11.09	184,017	1.07	13,982	100.18	100.35 ± 0.42
02	11.10	185,416	1.07	14,053	100.94	
03	11.09	184,694	1.08	14,080	100.54	
04	11.09	182,785	1.08	13,831	99.50	
05	11.09	183,933	1.08	13,876	100.13	
06	11.09	185,175	1.06	13,567	100.81	
<b>Average</b>	<b>11.09</b>	<b>184,337</b>	<b>1.07</b>	<b>13,898</b>	<b>100.35</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.52</b>	<b>N/A</b>	<b>N/A</b>	<b>0.53</b>	



(d)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.80	3,220,069	1.29	13,359	99.84	99.92 ± 0.14
02	3.81	3,233,931	1.29	13,333	100.27	
03	3.80	3,222,172	1.29	13,253	99.90	
04	3.80	3,218,170	1.29	13,353	99.78	
05	3.80	3,220,673	1.28	13,394	99.86	
06	3.80	3,221,575	1.29	13,282	99.89	
<b>Average</b>	<b>3.80</b>	<b>3,222,765</b>	<b>1.29</b>	<b>13,329</b>	<b>99.92</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.18</b>	<b>N/A</b>	<b>N/A</b>	<b>0.18</b>	

**Table 10.** Robustness-Flow rate. (a) Flow rate set 0.9 ml/min (Bimatoprost). (b) Flow rate set 0.9 ml/min (Timolol). (c) Flow rate set 1.1 ml/min (Bimatoprost). (d) Flow rate set 1.1 ml/min (Timolol).

(a)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	12.00	204,859	1.06	15,036	100.04	100.41 ± 0.21
02	11.99	205,377	1.08	14,826	100.29	
03	12.00	206,115	1.07	15,234	100.65	
04	11.99	206,243	1.05	15,190	100.72	
05	12.00	205,290	1.07	15,007	100.25	
06	12.00	205,798	1.06	15,299	100.50	
<b>Average</b>	<b>12.00</b>	<b>205,614</b>	<b>1.06</b>	<b>15,099</b>	<b>100.41</b>	
<b>% RSD</b>	<b>0.03</b>	<b>0.26</b>	<b>N/A</b>	<b>N/A</b>	<b>0.26</b>	

(b)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	4.18	3,564,375	1.30	14,401	99.74	99.96 ± 0.18
02	4.18	3,579,648	1.30	14,328	100.17	
03	4.18	3,566,820	1.30	14,284	99.81	
04	4.18	3,583,375	1.30	14,213	100.27	
05	4.18	3,565,396	1.30	14,163	99.77	
06	4.18	3,573,724	1.30	14,184	100.00	
<b>Average</b>	<b>4.18</b>	<b>3,572,223</b>	<b>1.30</b>	<b>14,262</b>	<b>99.96</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.22</b>	<b>N/A</b>	<b>N/A</b>	<b>0.22</b>	

(c)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	9.84	166,503	1.06	13,234	99.44	100.17 ± 0.30
02	9.84	168,231	1.08	13,330	100.47	
03	9.84	168,043	1.06	13,290	100.36	
04	9.84	167,874	1.06	13,283	100.26	
05	9.84	167,922	1.07	13,427	100.29	
06	9.84	167,786	1.07	13,101	100.20	
<b>Average</b>	<b>9.84</b>	<b>167,726</b>	<b>1.07</b>	<b>13,277</b>	<b>100.17</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.37</b>	<b>N/A</b>	<b>N/A</b>	<b>0.37</b>	

(d)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.43	2,914,455	1.28	13,187	100.02	100.06 ± 0.09
02	3.43	2,919,415	1.28	13,215	100.19	
03	3.43	2,912,846	1.28	13,083	99.96	
04	3.43	2,916,086	1.28	13,060	100.07	
05	3.43	2,911,975	1.28	13,140	99.93	
06	3.43	2,918,578	1.28	13,019	100.16	
<b>Average</b>	<b>3.43</b>	<b>2,915,559</b>	<b>1.28</b>	<b>13,117</b>	<b>100.06</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.10</b>	<b>N/A</b>	<b>N/A</b>	<b>0.11</b>	

**Table 11.** Robustness-pH of mobile phase. (a) pH 2.7 (Bimatoprost). (b) pH 2.7 (Timolol). (c) pH 2.9 (Bimatoprost). (d) pH 2.9 (Timolol).

(a)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	10.36	184,535	1.06	13,947	99.90	99.73 ± 0.19
02	10.36	183,480	1.07	14,157	99.33	
03	10.36	184,471	1.07	13,815	99.86	
04	10.36	183,855	1.06	14,128	99.53	
05	10.36	184,580	1.08	14,293	99.92	
06	10.37	184,403	1.08	14,375	99.83	
<b>Average</b>	<b>10.36</b>	<b>184,221</b>	<b>1.07</b>	<b>14,119</b>	<b>99.73</b>	
<b>% RSD</b>	<b>0.03</b>	<b>0.24</b>	<b>N/A</b>	<b>N/A</b>	<b>0.24</b>	

(b)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.76	3,209,495	1.29	13,722	99.82	99.80 ± 0.06
02	3.76	3,206,902	1.29	13,625	99.74	
03	3.76	3,210,173	1.29	13,602	99.84	
04	3.76	3,204,195	1.29	13,736	99.65	
05	3.76	3,210,948	1.29	13,534	99.86	
06	3.76	3,210,956	1.29	13,566	99.86	
<b>Average</b>	<b>3.76</b>	<b>3,208,778</b>	<b>1.29</b>	<b>13,631</b>	<b>99.80</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.08</b>	<b>N/A</b>	<b>N/A</b>	<b>0.08</b>	

(c)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	10.36	184,535	1.06	13,947	99.90	99.73 ± 0.19
02	10.36	183,480	1.07	14,157	99.33	
03	10.36	184,471	1.07	13,815	99.86	
04	10.36	183,855	1.06	14,128	99.53	
05	10.36	184,580	1.08	14,293	99.92	
06	10.37	184,403	1.08	14,375	99.83	
<b>Average</b>	<b>10.36</b>	<b>184,221</b>	<b>1.07</b>	<b>14,119</b>	<b>99.73</b>	
<b>% RSD</b>	<b>0.03</b>	<b>0.24</b>	<b>N/A</b>	<b>N/A</b>	<b>0.24</b>	

(d)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.74	3,217,642	1.29	13,653	99.69	99.64 ± 0.11
02	3.74	3,213,648	1.29	13,545	99.56	
03	3.74	3,217,999	1.29	13,573	99.70	
04	3.73	3,220,173	1.29	13,773	99.77	
05	3.74	3,207,964	1.29	13,521	99.39	
06	3.74	3,218,668	1.29	13,600	99.72	
<b>Average</b>	<b>3.74</b>	<b>3,216,016</b>	<b>1.29</b>	<b>13,611</b>	<b>99.64</b>	
<b>% RSD</b>	<b>0.02</b>	<b>0.14</b>	<b>N/A</b>	<b>N/A</b>	<b>0.14</b>	

**Table 12.** Robustness-Mobile phase composition variation. (a) Buffer: Organic = 48:52 (Bimatoprost). (b) Buffer: Organic = 48:52 (Timolol). (c) Buffer: Organic = 52:48 (Bimatoprost). (d) Buffer: Organic = 52:48 (Timolol).

(a)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	8.81	184,562	1.10	14,474	99.09	99.82 ± 0.77
02	8.82	186,052	1.08	13,994	99.89	
03	8.81	187,691	1.09	13,621	100.77	
04	8.82	185,065	1.07	13,831	99.36	
05	8.78	183,795	1.09	13,475	98.68	
06	8.79	188,317	1.08	13,144	101.11	
<b>Average</b>	<b>8.80</b>	<b>185,914</b>	<b>1.09</b>	<b>13,756</b>	<b>99.82</b>	
<b>% RSD</b>	<b>0.16</b>	<b>0.96</b>	<b>N/A</b>	<b>N/A</b>	<b>0.96</b>	
(b)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.59	3,216,037	1.29	12,959	99.57	99.41 ± 0.12
02	3.59	3,208,620	1.29	12,872	99.34	
03	3.59	3,212,021	1.29	13,017	99.44	
04	3.59	3,212,973	1.29	13,140	99.47	
05	3.58	3,213,199	1.29	13,225	99.48	
06	3.58	3,201,721	1.29	12,978	99.13	
<b>Average</b>	<b>3.59</b>	<b>3,210,762</b>	<b>1.29</b>	<b>13,032</b>	<b>99.41</b>	
<b>% RSD</b>	<b>0.14</b>	<b>0.16</b>	<b>N/A</b>	<b>N/A</b>	<b>0.15</b>	
(c)						
Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	12.08	185,239	1.09	14,162	100.99	100.34 ± 0.58
02	12.09	182,773	1.08	14,205	99.64	
03	12.09	184,794	1.05	14,547	100.74	
04	12.07	185,623	1.08	14,032	101.19	
05	12.09	183,510	1.08	14,413	100.04	
06	12.09	182,416	1.06	14,539	99.45	
<b>Average</b>	<b>12.08</b>	<b>184,059</b>	<b>1.07</b>	<b>14,316</b>	<b>100.34</b>	
<b>% RSD</b>	<b>0.08</b>	<b>0.73</b>	<b>N/A</b>	<b>N/A</b>	<b>0.73</b>	

(d)

Injection Number.	Retention Time	Area	Tailing Factor	Theoretical Plate	% of Assay	95% Confidence Interval
01	3.88	3,214,261	1.30	12,998	100.05	100.25 ± 0.27
02	3.88	3,223,492	1.29	12,844	100.34	
03	3.88	3,227,900	1.30	12,676	100.48	
04	3.87	3,235,866	1.30	13,051	100.73	
05	3.88	3,216,075	1.30	12,864	100.11	
06	3.88	3,204,804	1.29	13,142	99.76	
<b>Average</b>	<b>3.88</b>	<b>3,220,400</b>	<b>1.30</b>	<b>12,929</b>	<b>100.25</b>	
<b>% RSD</b>	<b>0.05</b>	<b>0.34</b>	<b>N/A</b>	<b>N/A</b>	<b>0.34</b>	

### 6.2.9. Forced Degradation Studies

The forced degradation results showed that the Bimatoprost and Timolol drug was liable to acid and degraded to about 10.94% for Bimatoprost & 10.32% for Timolol. In basic degradation, it was found to be degraded to about 5.95% for Bimatoprost & 12.22% for Timolol. Upon oxidation, it was degraded to about 9.02% for Bimatoprost & 19.21% for Timolol and in thermal degradation to about 17.61% for Bimatoprost & 1.49% for Timolol. Very less degradation was observed in photolytic degradation to about 4.99% for Bimatoprost & 13.04% for Timolol. For the validation of any chromatographic assay, 5% to 20% of degradation is acceptable [12].

Therefore, in the present method, the % degraded amount is within the specified limits and moreover, the successful separation of active pharmaceutical products from degradation products without any interference proved the stability indicating the nature of the method and also proves that there was no influence of temperature on the performance of the newly developed method. The obtained chromatograms during stress testing were depicted in **Figures 2-6**. The measured values are provided in **Table 13**.

## 7. Conclusions

The present established stability indicates the Reverse phased High performance liquid chromatographic method is new, fast, and easier to quantify the Bimatoprost and Timolol drug with precise and accurate results. The successful separation of all the degradation products from the active pharmaceutical ingredient proved the specificity and the stability indicating the nature of the developed method. In comparison to the reported method for Bimatoprost and Timolol separate method validation study, this method is more perfect for Bimatoprost and Timolol combination ophthalmic preparation. In addition to that available reported methods are not having stability indications for combination preparation of Bimatoprost and Timolol ophthalmic solution. The run time of the

**Table 13.** Force degradation.

Treatment condition	Sample Name	% of recovery	Remarks
Without Treatment	Placebo	N/A	Not degraded
	Sample	Bimatoprost: 99.60% and Timolol: 99.84%	Not degraded
Acid hydrolysis of Bimatoprost (treated with 0.2 ml of 1N HCl at 85° for 15 min)	Placebo	N/A	Not degraded
	Standard	88.21	Bimatoprost peak is degraded
	Sample	88.66	Bimatoprost peak is degraded
Acid hydrolysis of Timolol (treated with 1ml of 1N HCl at 85° for 11 hours)	Placebo	N/A	Not degraded
	Standard	89.79	Timolol peak is degraded
	Sample	89.52	Timolol peak is degraded
Base hydrolysis of Bimatoprost (treated with 10 ml of 1N NaOH at 85° for 1.5 hours)	Placebo	N/A	Not degraded
	Standard	93.00	Bimatoprost peak is degraded;
	Sample	93.65	Bimatoprost peak is degraded;
Base hydrolysis of Timolol (treated with 0.5 ml of 1N NaOH at 85° for 1.5 hours)	Placebo	N/A	Not degraded
	Standard	87.55	Timolol peak is degraded
	Sample	87.62	Timolol peak is degraded
Peroxide oxidation of Bimatoprost (0.5 ml of 30% H <sub>2</sub> O <sub>2</sub> heated at 85°C for 50 minutes)	Placebo	N/A	Not degraded
	Standard	90.35	Bimatoprost peak is degraded
	Sample	90.58	Bimatoprost peak is degraded
Peroxide oxidation of Timolol (0.5 ml of 30% H <sub>2</sub> O <sub>2</sub> heated at 85°C for 15 minutes)	Placebo	N/A	Not degraded
	Standard	79.38	Timolol peak is degraded
	Sample	79.63	Timolol peak is degraded
Thermal degradation of Bimatoprost heat at 85°C for 4.5 hours	Placebo	N/A	Not degraded
	Standard	82.26	Bimatoprost peak is degraded
	Sample	81.99	Bimatoprost peak is degraded
Thermal degradation of Timolol (heat at 85°C for 19 hours)	Placebo	N/A	Not degraded
	Standard	98.18	Timolol peak is not degraded
	Sample	98.35	Timolol peak is not degraded
Photolytic degradation of Bimatoprost (exposed to direct sunlight for 02 hours)	Placebo	N/A	Not degraded
	Standard	94.07	Bimatoprost peak is degraded
	Sample	94.61	Bimatoprost peak is degraded
Photolytic degradation of Timolol (exposed to direct sunlight for 02 hours)	Placebo	N/A	Not degraded
	Standard	86.64	Timolol peak is degraded
	Sample	86.80	Timolol peak is degraded

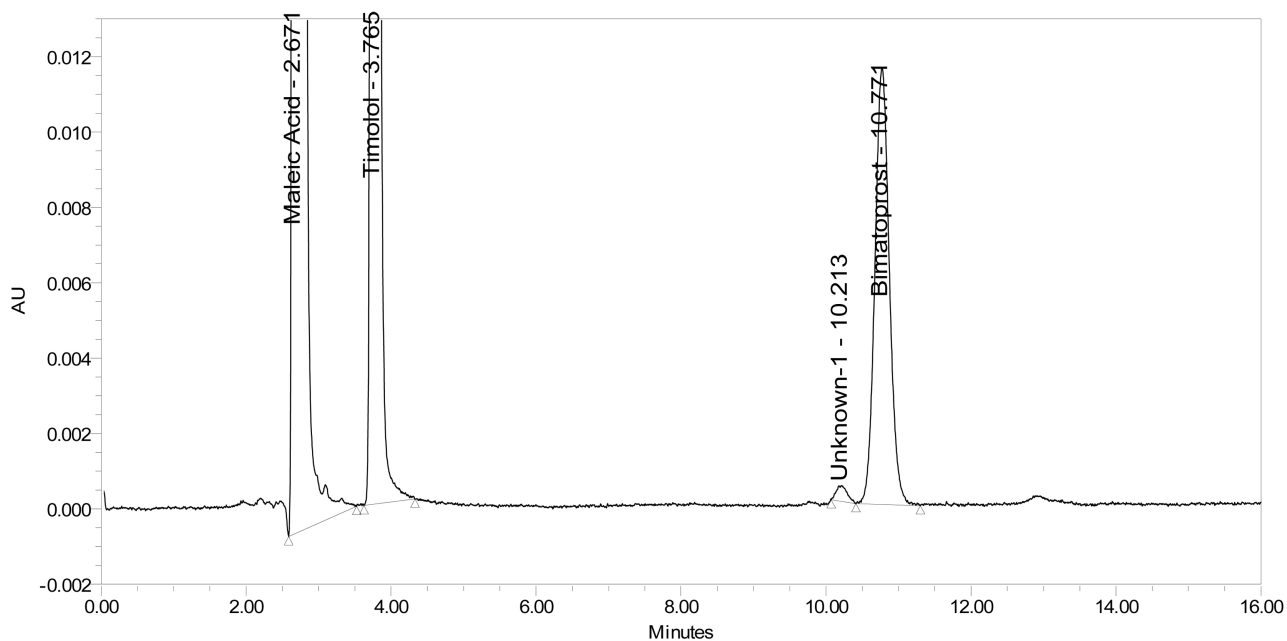


Figure 3. Acid degradation chromatogram of Bimatoprost and Timolol.

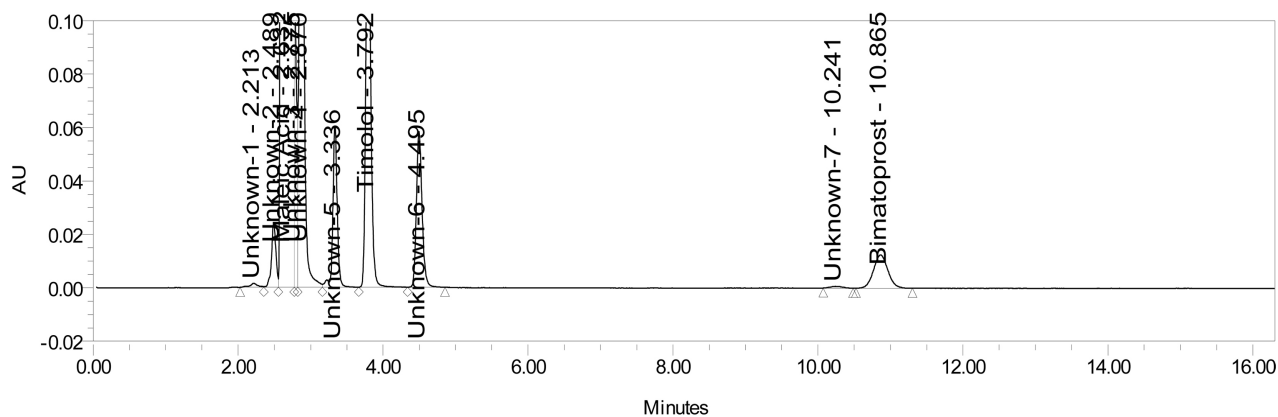


Figure 4. Basic degradation chromatogram of Bimatoprost and Timolol.

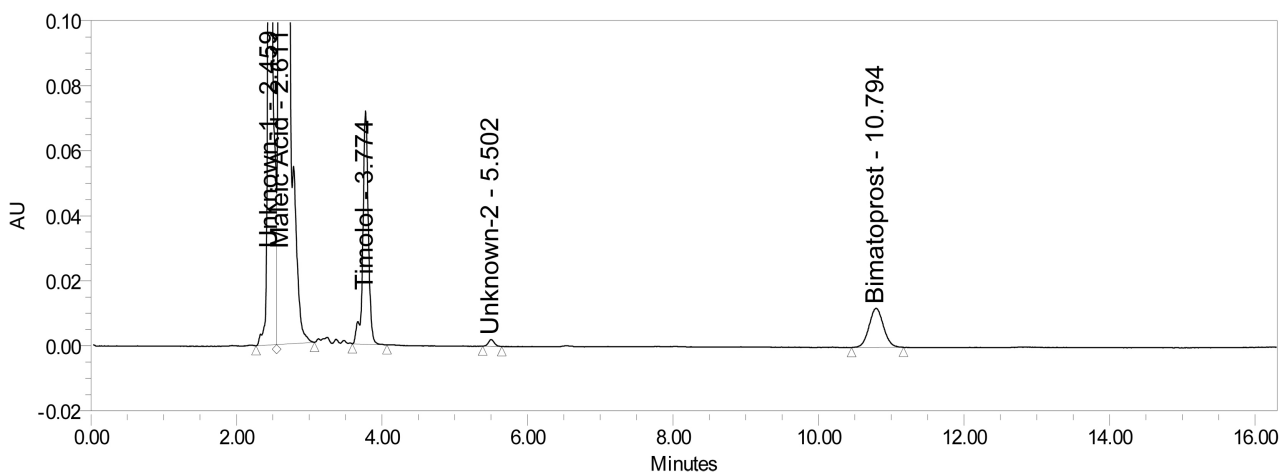
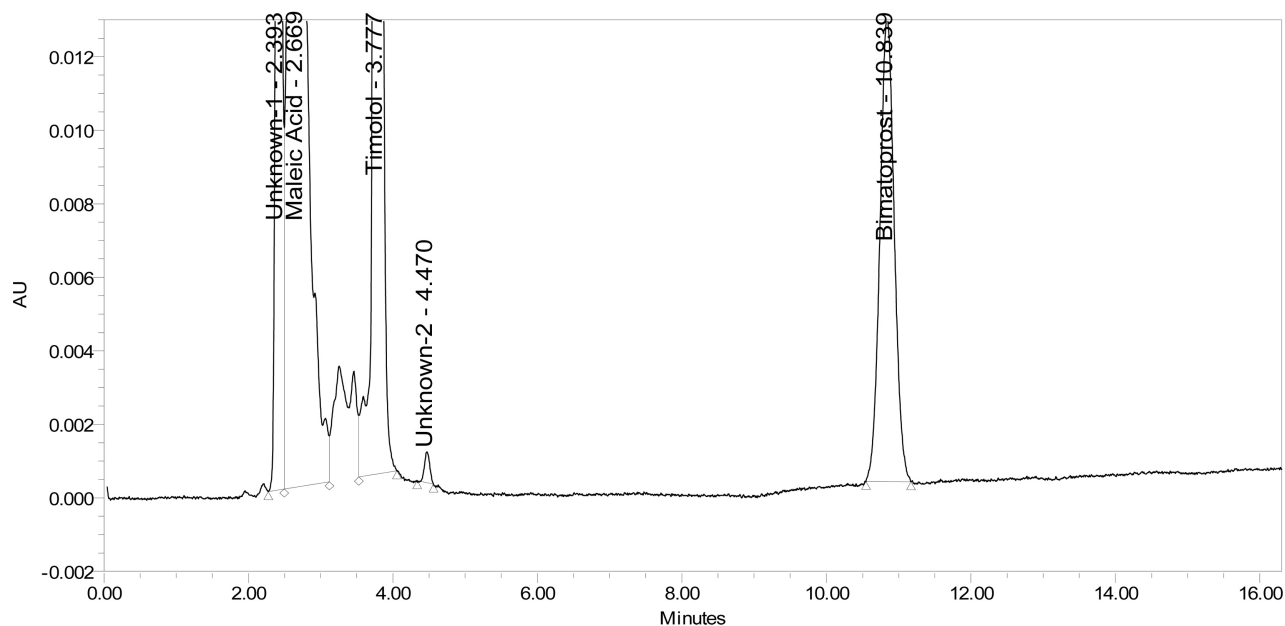


Figure 5. Oxidative degradation chromatogram of Bimatoprost and Timolol.



**Figure 6.** Thermal degradation chromatogram of Bimatoprost and Timolol.

method is 10.81 min for Bimatoprost and 3.77 min for Timolol which proves that this is a faster stability indication method with common column and mobile phase.

Thus, the shorter duration of analysis time, more sensitivity, and cost-effectiveness revealed that it is suitably applied for routine laboratory use for regular testing and also stability testing of pharmaceutical ophthalmic products.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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