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Dr. Harmeet Kaur Kohli
Principal author,
Associate Professor,
Department of Chemistry,
G.N. Khalsa College of Arts,
Science and Commerce
(Autonomous), Matunga,
Mumbai, Maharashtra, India

Satish K Ghumare
Department of Chemistry, G.
N. Khalsa College of Arts,
Science and Commerce
(Autonomous), Matunga,
Mumbai, Maharashtra, India

Dr. Deepa Parab
Associate Professor,
Department of Chemistry,
G.N. Khalsa College of Arts,
Science and Commerce
(Autonomous), Matunga,
Mumbai, Maharashtra, India

Vijay A Bagul
The Institute of Science, Dr.
Homi Bhabha State
University, Madam Kama
Road, Mumbai, Maharashtra,
India

Corresponding Author:
Dr. Deepa Parab
Associate Professor,
Department of Chemistry,
G.N. Khalsa College of Arts,
Science and Commerce
(Autonomous), Matunga,
Mumbai, Maharashtra, India

Analytical method development, validation and forced degradation study of timolol maleate and brinzolamide in ophthalmic suspension

Harmet Kaur Kohli, Satish K Ghumare, Deepa Parab and Vijay A Bagul

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Abstract

In the present work, a stability-indicating reverse phase HPLC (RP-HPLC) technique using an AD HOC Prudent C18 (125 mm x 4.6 mm; 5 μ) P/N:P18-12540-5 column was developed and subsequently validated for the detection of Timolol Maleate and Brinzolamide simultaneously. ICH guidelines were followed for the method's validation. All system suitability parameters were determined to be within the intended ranges. This method was found to be specific, precise, linear, accurate and robust. Forced degradation studies were carried out under various circumstances. The drug molecules were not impacted during stress application in thermal and acidic conditions. This method will be beneficial for qualitative as well quantitative determination of Timolol and Brinzolamide used in combination in process and final formulation.

Keywords: RP-HPLC, simultaneous analytical validation, ICH guidelines, forced degradation

Introduction

Timolol maleate is a nonselective beta-adrenergic antagonist drug which is either used in the oral form or as eye drops for the treatment of hypertension, heart attack and glaucoma^[1]. Its IUPAC name is (2S)-1-(tert-butylamino)-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol; (2Z)-but-2-enedioic acid^[2].

Figure. 1a. In the oral dosage form, this medicine is used to manage high blood pressure^[3, 4]. These auxiliary drugs to lower the intraocular pressure may not be used or may be used together with this preparation as eye drops for those suffering open angle glaucoma^[5-7] or ocular hypertension as an eye preparation.

Brinzolamide is a carbonic anhydrase II inhibitor^[8, 9]. When used as eye drops, it acts as an ocular hypertension reducing agent. Its IUPAC name is (4R)-4-(Ethylamino)-2-(3-methoxypropyl)-3,4-dihydro-2H-thieno[3,2-e][1,2]thiazine-6-sulfonamide 1,1-dioxide².

Figure. 1b. In the Ophthalmic dosage form it works by decreasing aqueous humor secretion which results in lowering the intra-ocular pressure in the interior part of eye^[10]. The combined effect of these two drugs plays a vital role for Glaucoma treatment and shows more efficiency in lowering the intra ocular pressure (IOP) as compared to separate drug doses^[11]. The authors describe several methods for the evaluation of timolol and brinzolamide^[12-14]. However, there is no established rapid and reliable analytical method required for the identification and subsequent quantification of these two drugs for use in eye preparation in combination dosage form. We have demonstrated that a combination drug formulation can be analyzed rapidly and accurately employing a very sensitive (Stability indicating)^[19, 20] RP-HPLC technique developed and validated in this work.

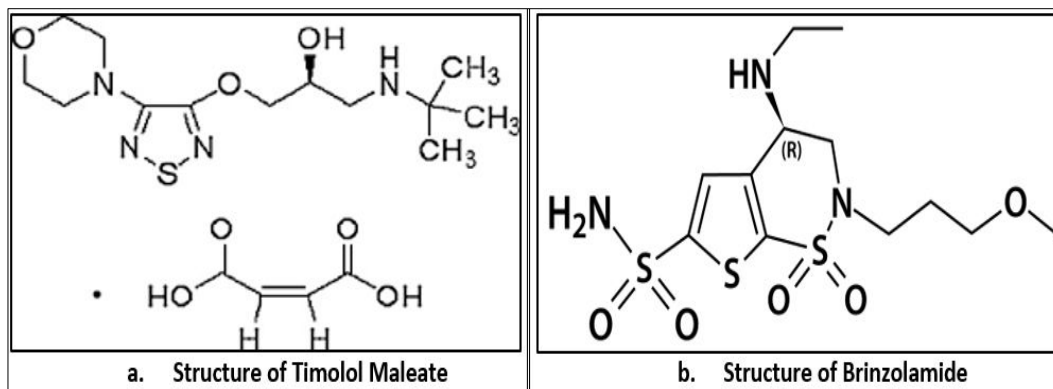


Fig 1: Structure of TIM and BRZ

Materials and Methods

Chemicals and reagents

Timolol maleate standard and Brinzolamide standard were received from Global Calcium Ltd as free samples. HPLC grade (Milli Q) water, acetonitrile (HPLC grade - Merck) and sodium acetate salt (Make - Rankem) were purchased from the open market.

Instrument and system

The instruments used for the analysis were PDA or UV-visible Detection (Ultimate 3000), analytical balance (Sartorius), pH meter (Labindia), ultrasonic bath (Precisonic), and chromatographic system: Thermo Scientific HPLC with Chromeleon Software version 7.2 and separation module. The details are given in Table 1.

Mobile phase preparation

The addition of 5.77 g of sodium acetate and 1.78 g of acetic acid to 800 mL of HPLC grade (Milli Q) water gave 0.1 M sodium acetate solution (buffer pH 4.25). The pH was adjusted to 4.25 with the help of 10N HCl. The final volume was made to 1L with HPLC grade (Milli Q) water. 70: 30 v/v of acetate buffer solution of pH 4.25 and acetonitrile were mixed properly and filtered through 0.45µ nylon membrane filter and degassed to give the mobile phase.

Standard preparation

Timolol Maleate weighing 34 mg and Brinzolamide Standard weighing 25 mg were then placed in a 100 mL and a 50 mL volumetric flask respectively. These volumetric flasks were topped with a mobile phase of 30 mL and the medications dissolved by sonication. The proper level of clear solutions was achieved through dilution with the same mobile phase. The volumetric flasks of 50 mL of content from five mL of the resulting solutions were quantitatively filled using the same mobile phase. The final solutions contained respectively 25 µg/mL Timolol – 25 ppm and 50µg/mL Brinzolamide – 50 ppm.

Sample preparation

The test sample medicine, Brinzolamide and Timolol in suspension form (Brincox T eye drops) manufactured by Ajanta Pharma was procured from the Mumbai open market. Sample solutions density (Weight per milliliter) was

determined by a densitometer and the value was utilized in the calculations. A 100 mL standard volumetric flask was prepared into which 0.5g of the sample solution was placed. In this case, following dilution with a further 60 mL of the diluent (mobile phase) was added, the flask was sonicated for five minutes, under occasional intermediate shaking. The suspension was subsequently allowed to standard temperature and pressure conditions. The solution after dilution up to the suitable levels with the mobile phase, shaken well and filtered through 0.45 µ nylon membrane syringe filter. The resulting solution contained 25 µg/mL of Timolol and 50 µg/mL of Brinzolamide respectively.

System suitability

The system suitability for Timolol and Brinzolamide was confirmed by evaluating the performance criteria during the analysis. The performance criteria involved achieving theoretical plates count not lower than 1000, tailing factor not exceeding 2.0 with RSD not exceeding 2.0%.

Method Development

In order to obtain the greatest possible separation between timolol and brinzolamide peaks several experiments were done with the use of buffers and stationary phases in a mobile phase [15-18]. The flow rate selected was 1.0 mL/min as the mobile phase comprising of an acetate buffer solution, pH 4.25 and the increasing concentration of acetonitrile 70:30 v/v brought an acceptable resolution between the two peaks. The column with this combination of the two drugs was selected based upon the sharpness of the peaks, the resolution of the peaks and speed of movement of the peaks. The stationary phase selected was a Prudent C18 AD HOC (125 x 4.6 mm; 5u) P/N: P18-12540-5. Since both timolol and brinzolamide had optimum absorbance at a wavelength of 274 nm, this was the wavelength selected¹². From the results obtained Timolol and brinzolamide were found to have retention time of 1.7 and 2.5 minutes respectively. The optimal chromatographic parameters chosen in Table 1 have been prepared considering all the conditions described in previous paragraphs. This stability indicating [19, 20] method has been determined for the intended purpose after full validation processes following ICH guidelines [21] and the respective chromatographic parameters.

Table 1: Optimized HPLC Chromatographic condition

Parameters	Description
Column Dimension	C18, 125 mm × 4.6 mm; 5 μ m
Column Used	AD HOC Prudent C18
Flow (mL/min)	1.0 mL/min
Volume of Injection	5 μ L
Run Period	5 min
Detection wavelength	274 nm
Mobile Phase	Acetate buffer pH 4.25: Acetonitrile (70: 30) v/v
System	Isocratic
Column Temperature	45 °C
Auto sampler Temperature	25 °C

Developed Method Validation: The developed method was optimized and subjected to validation by utilizing general validation parameters acceptance criteria as per ICH guideline Q2(R2) and USP chapter <1225>. A brief detail of validation outcomes is described along with proper data and final outcome for each parameter.

Specificity

The developed method specificity was also evaluated. This was illustrated by the injection of the blank, standard and individual solutions and noting the interference at the Timolol and Brinzolamide retention times.

Preparation of the blank solution began with diluting the mobile phase (The diluent). To prepare the placebo, the same procedures were used as when the sample was being prepared. After that, it was monitored with the chromatographic HPLC apparatus.

Precision

The standards of Timolol and Brinzolamide were readied according to the prescription in standard preparation and injected in the HPLC system with six replicates. The percentage RSD of Timolol and Brinzolamide area was calculated.

Method Precision

The test method was followed and accordingly six individual sample preparations were prepared, injected in the system and the percentage RSD of these six assay results was calculated.

Intermediate Precision

Six different sample preparations are done as per the test procedure and exposed to various HPLC systems, columns and using different lot of chemicals. The experiment was conducted on different days. Six assay results were used to calculate the % RSD. The two assay datasets were also compared using method precision and intermediate precision to assess the percent absolute difference.

Linearity and Range

When the test concentration data is directly proportional to the analyte concentration of the sample within a certain range the analytical method is said to be linear.

The concentration 50% to 150% was taken as the standard concentration to determine linearity and range. The graph of

concentration taken in μ g/mL (x-axis) was plotted against the response (Observed area) (y-axis). Correlation coefficient, percentage y-intercept, slope of regression line, residual sum of squares and percentage relative standard deviation (RSD) of areas of Timolol and Brinzolamide respectively were calculated

The concentrations of the standard solutions were prepared at 50%, 80%, 100%, 120% and 150% of the target concentration of Timolol (25 μ g/mL) and Brinzolamide (50 μ g/mL) respectively. These five levels covered lower and higher level method range.

Accuracy

To determine the accuracy of the analysis three target concentration levels were picked. A placebo was added to a standard stock solution at a concentration of 50%, 100% and 150% respectively in triplicate. As per procedure, these three levels were injected. The overall recovery across all SG levels was 98% to 102% range.

Intermediate precision (Ruggedness)

Although multiple equipment and study days were employed in the activity, the intermediate parameter similarly showed no impact on results. The percent relative standard deviation of the studied data were 0.90 and 0.30 for Timolol while for Brinzolamide 0.79 and 0.70 which were done on different days using separate instrument. The results are presented in Table 6.

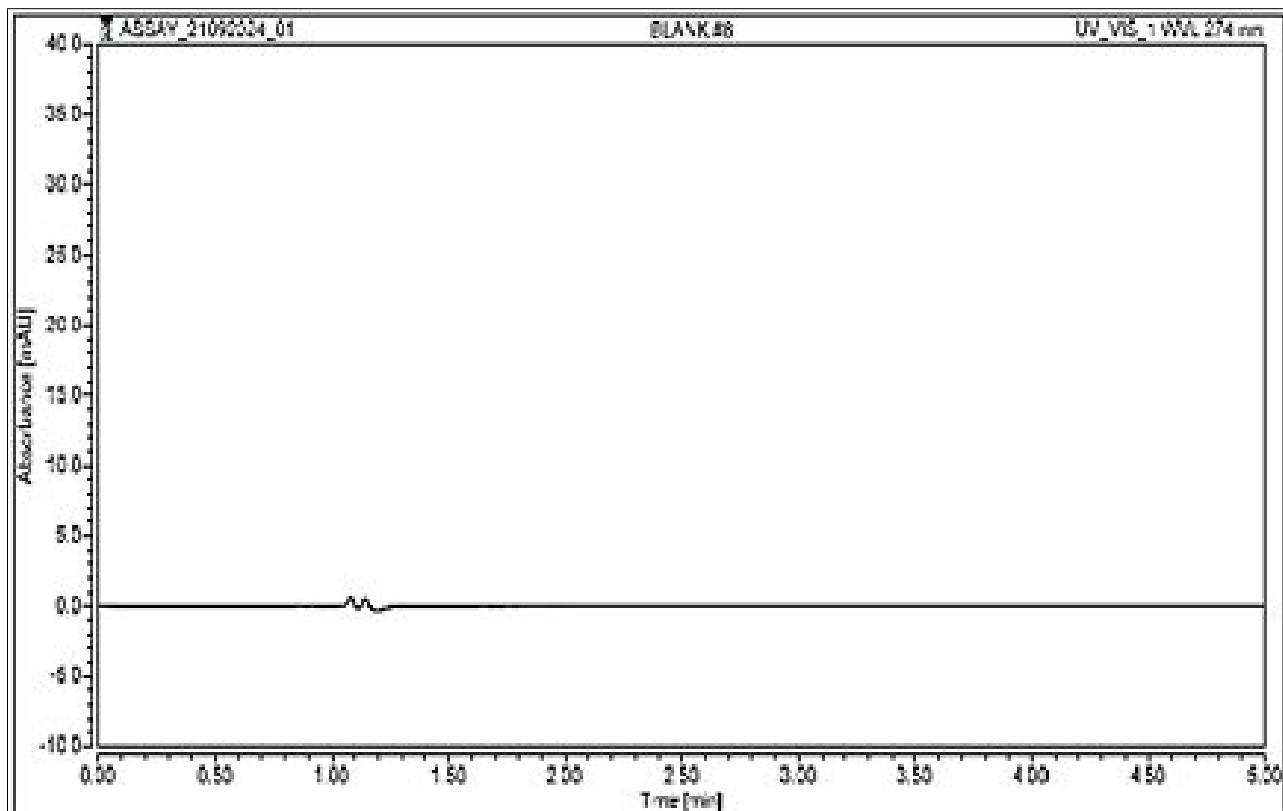
Robustness

The intentional adjustment (Robustness) of the analytical process and instrumental parameter was examined by small but deliberate changes in the analytical method which uninfluenced the performance of the method. The sample and standard were performed as per the intended methodology. The method variation parameters like flow change ± 0.1 mL of 1.0 mL (0.9 mL/min and 1.1 mL/min), column oven temperature ± 5 °C (40 °C and 50 °C) and mobile phase buffer pH ± 0.2 (pH 4.05 and pH 4.45) were performed.

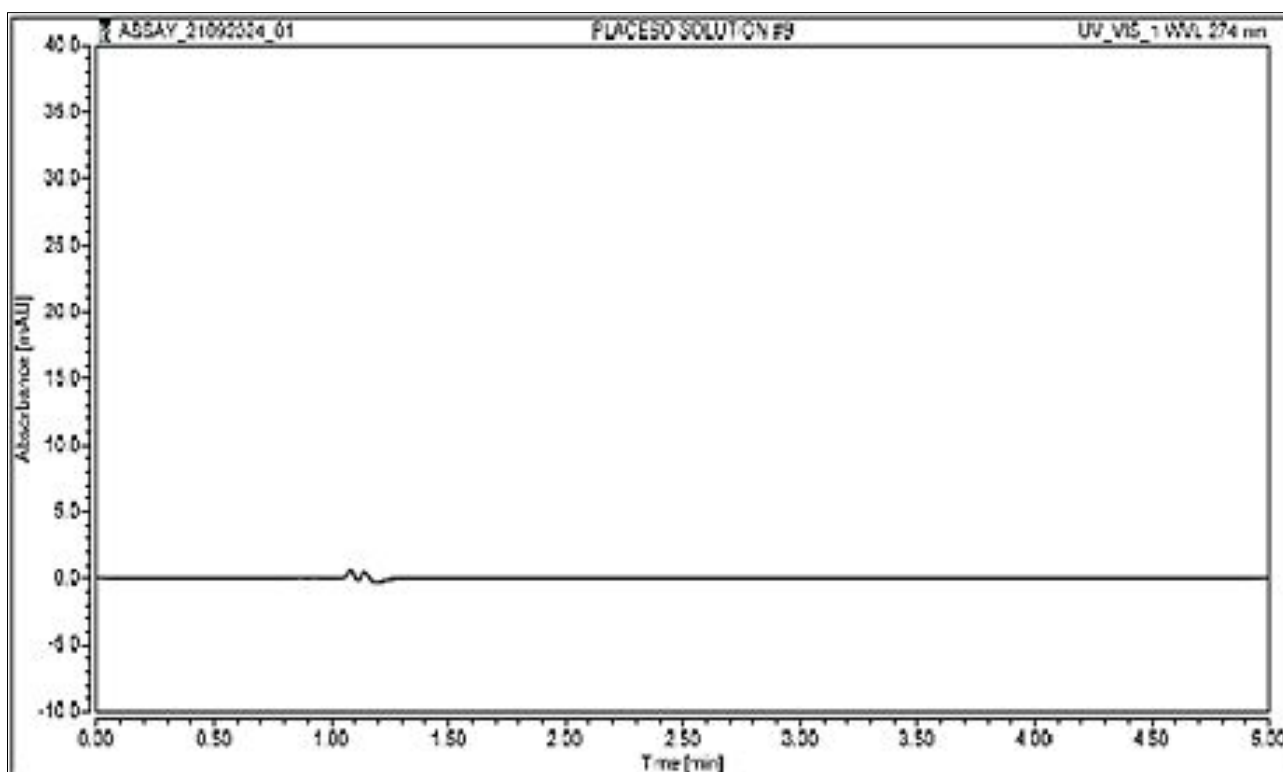
Results and Interpretation

Specificity

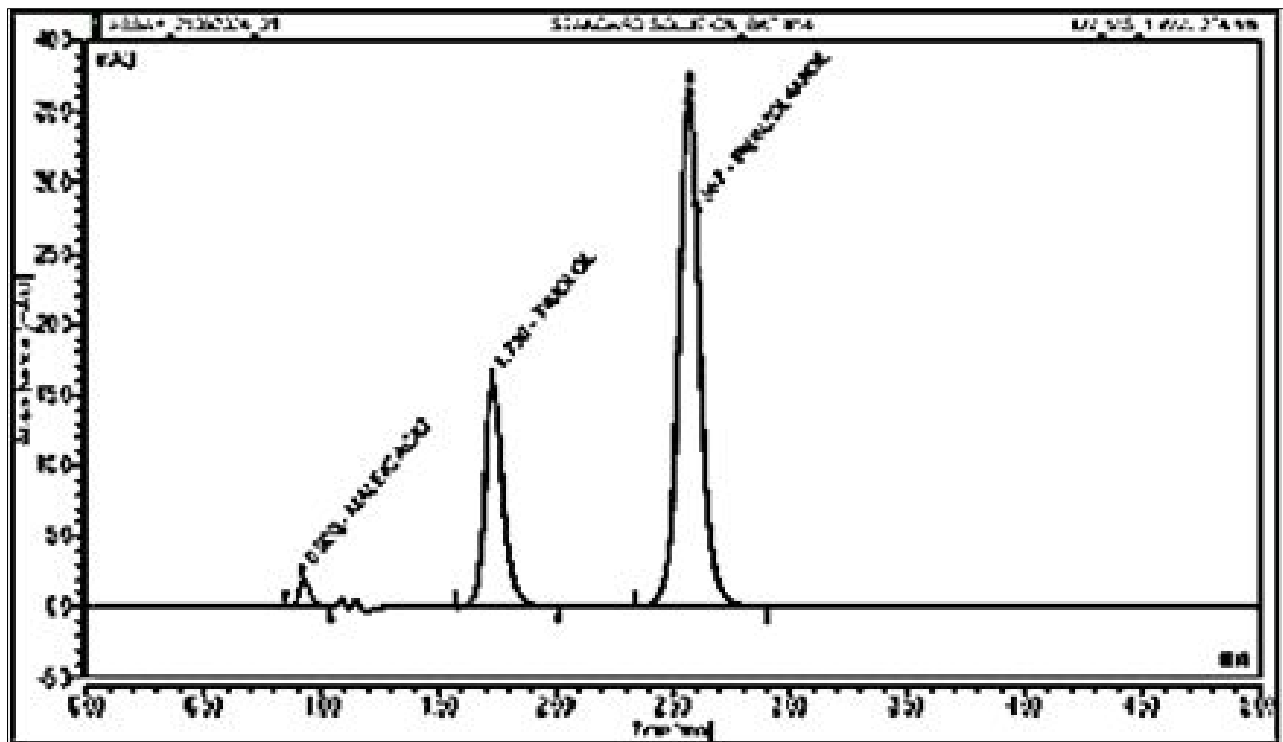
The blank and placebo showed no interference observance at the Timolol (Rt- 1.74 Minutes) and Brinzolamide (Rt- 2.5 Minutes) retention time respectively as per Figure 2.



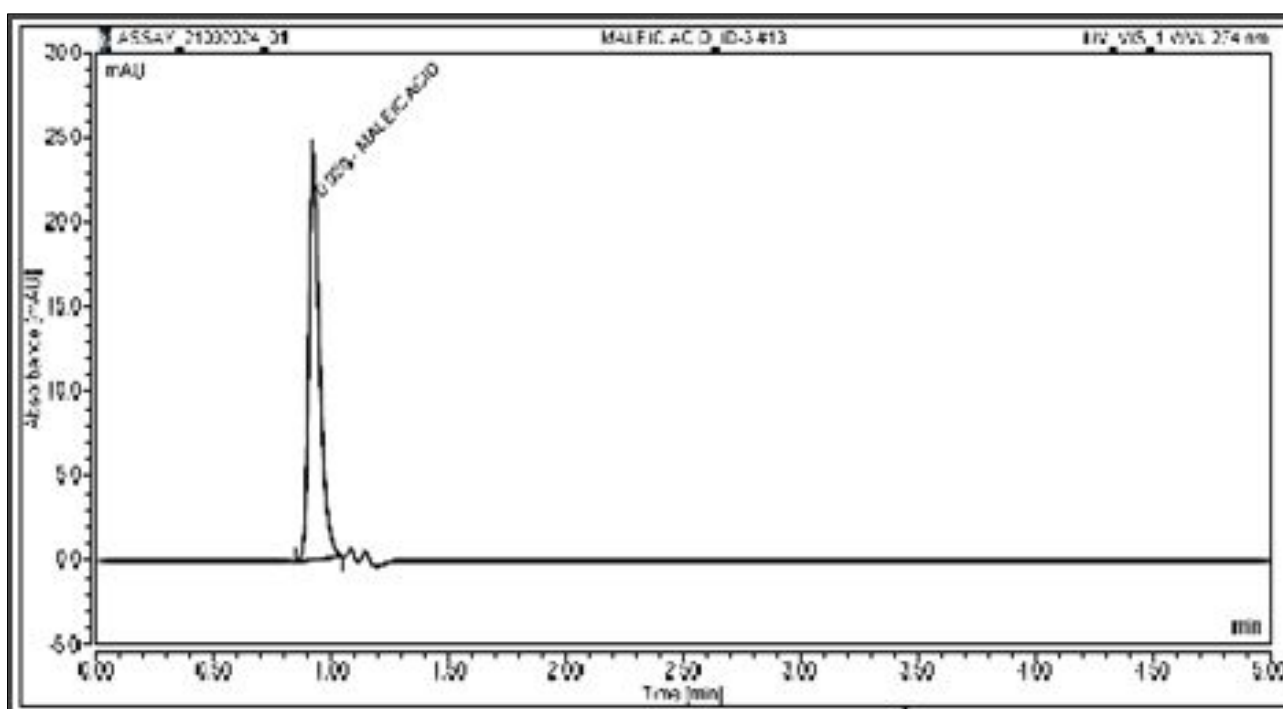
a. Blank Chromatogram



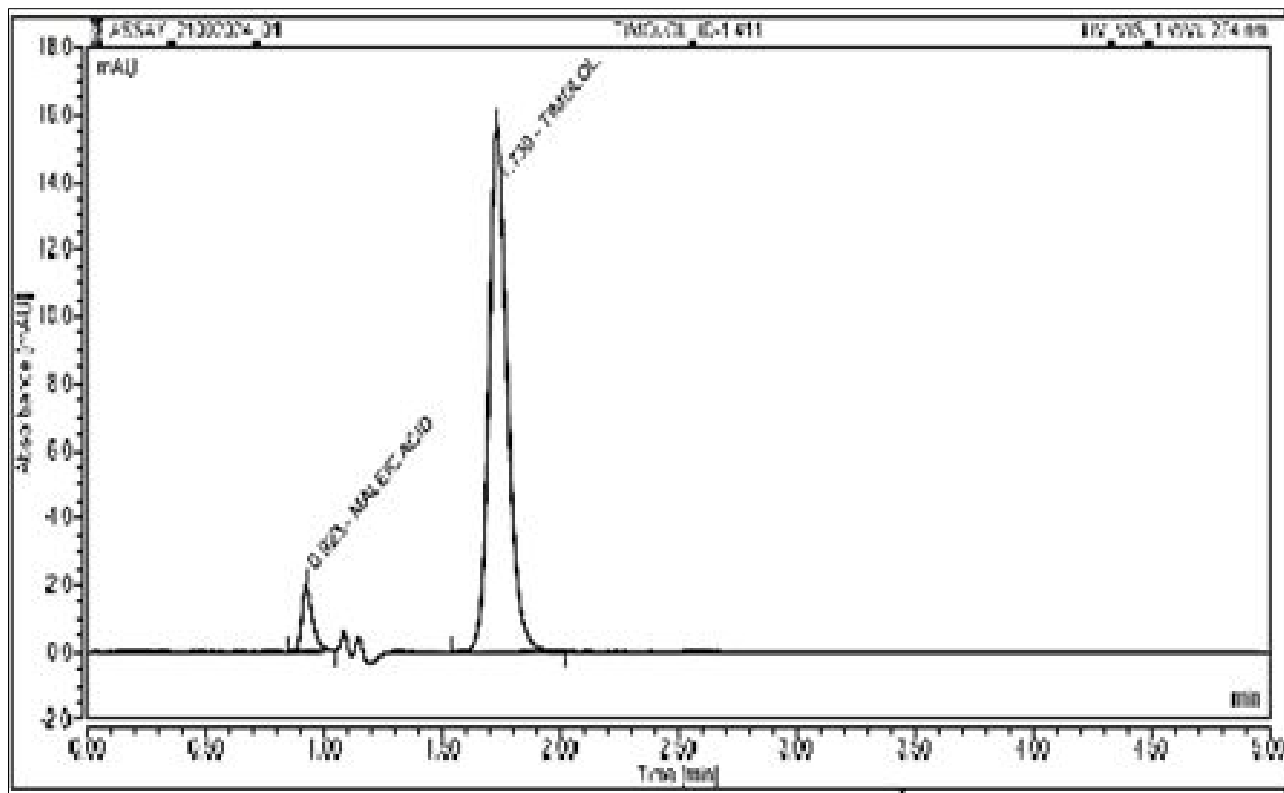
b. Placebo Chromatogram



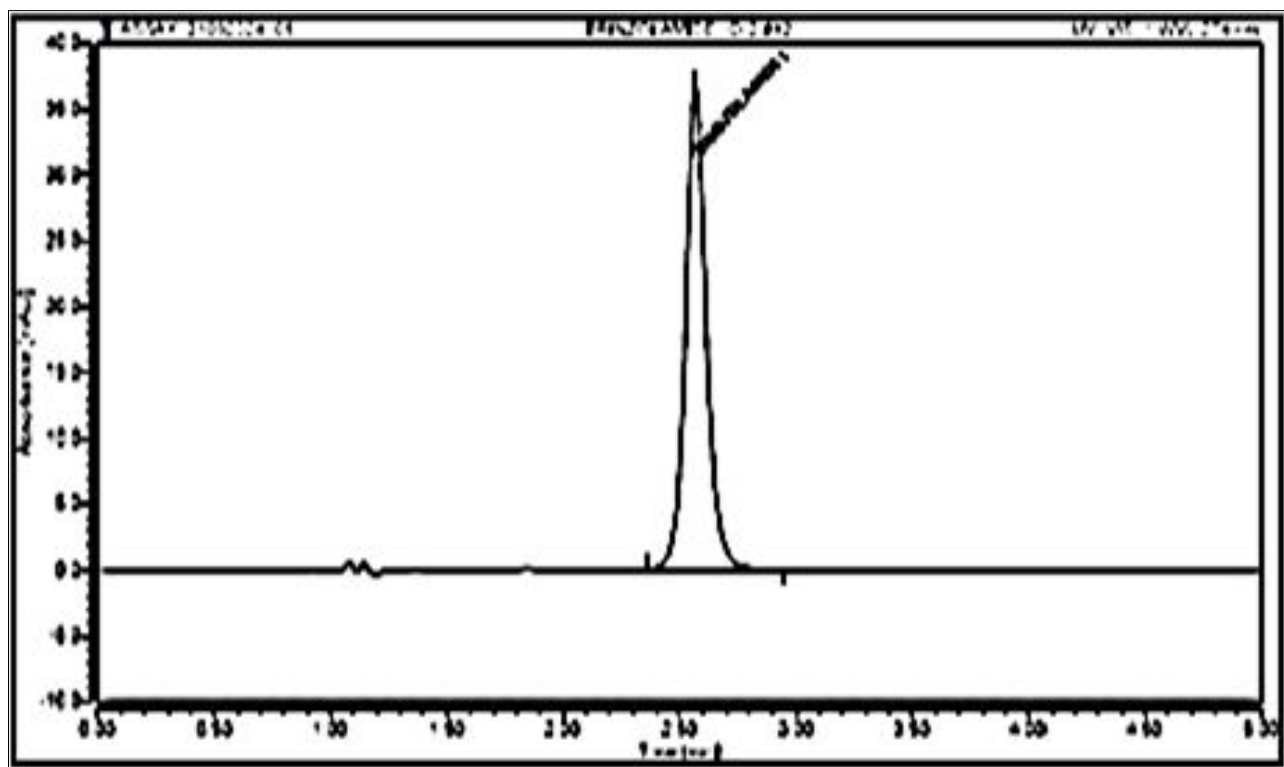
c. Standard Chromatograph



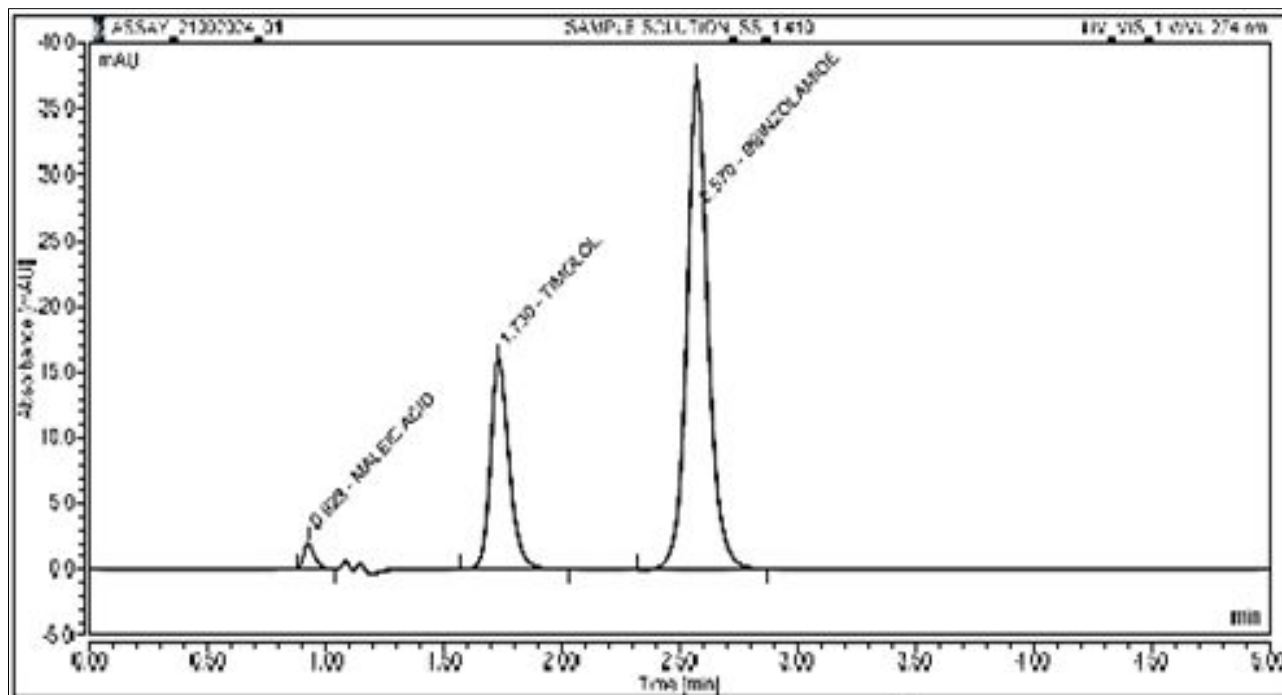
d. Individual maleic acid Chromatograph



e. Individual Timolol Chromatograph



f. Individual Brinzolamide Chromatograph

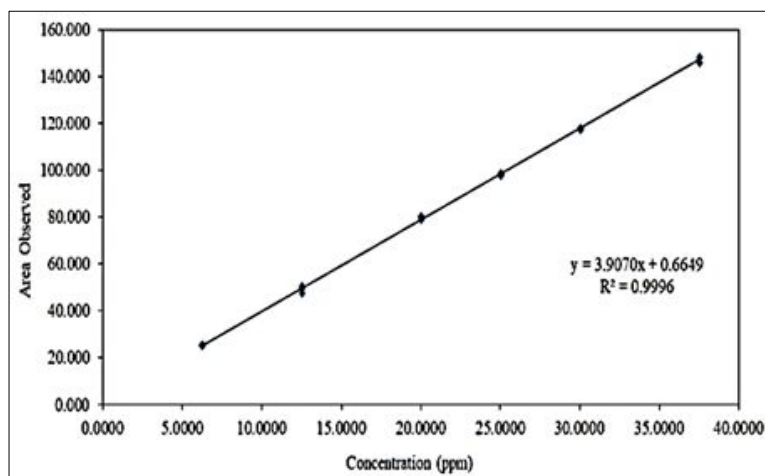
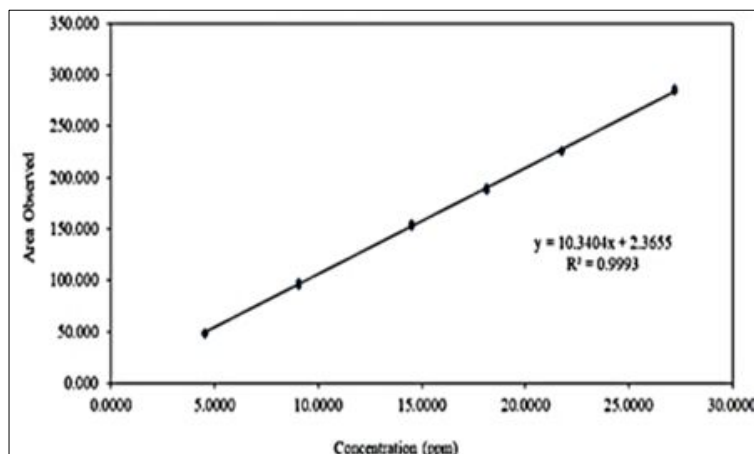


g. Sample Chromatograph

Fig 2: Specificity chromatograph of TIM and BRZ**Linearity and Range**

The R^2 value (correlation of coefficient) was observed at 0.9998 and 0.9955 respectively for Timolol and

Brinzolamide, which was within the limit (not less than 0.995) as per validation linearity criteria. The linearity graphs are shown in the Figure 3 and 4.

**Fig 3:** Linearity plot of Timolol**Fig 4:** Linearity plot of Brinzolamide

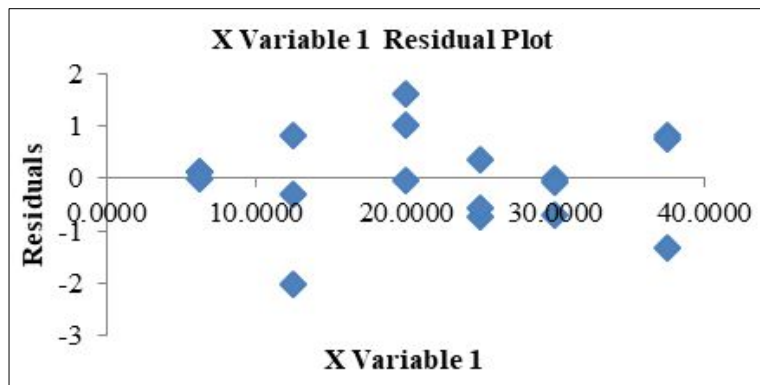


Fig 5: Residual plot of Timolol

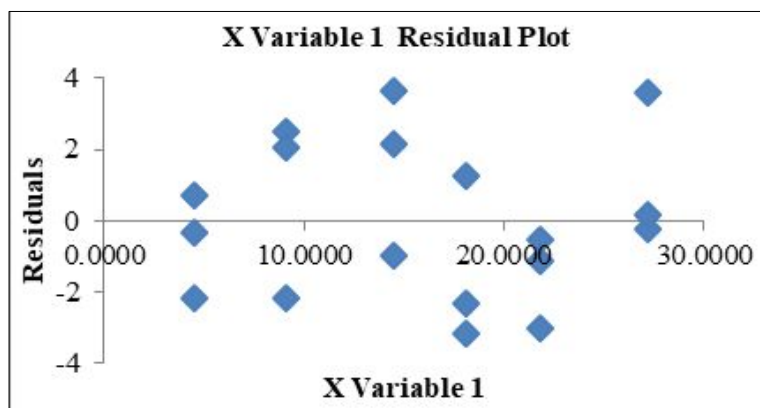


Fig 6: Residual plot of Brinzolamide

Table 2: Linearity summary for TIM and BRZ

Parameter for Linearity	Timolol	Brinzolamide	Criteria
Correlation coefficient R^2	0.9996	0.9993	> 0.999
%Y – axis intercept	0.84	1.55	$\leq \pm 3\%$
Slope of regression line	3.91	10.34	To be reported
Residual sum of squares	13.1	80.3	To be reported

Accuracy: The methods accuracy as measured by percentage recovery remained in the range of 98-102% (Table 3 and 4)

Table 3: Accuracy for Timolol

Accuracy Level	Concentration of standard added ($\mu\text{g}/\text{mL}$)	Concentration of standard recovered ($\mu\text{g}/\text{mL}$)	% Recovery	Average	% RSD
50%- 1	12.6018	12.6655	101	101	1.36
50%- 2	12.6018	12.5939	100		
50%- 3	12.6018	12.8850	102		
100%-1	25.2036	24.9870	99	100	0.68
100%-2	25.2036	25.2701	100		
100%-3	25.2036	25.0312	99		
150%-1	37.8054	37.8878	100	100	0.93
150%-2	37.8054	37.8684	100		
150%-3	37.8054	37.3389	99		

Table 4: Accuracy for Brinzolamide

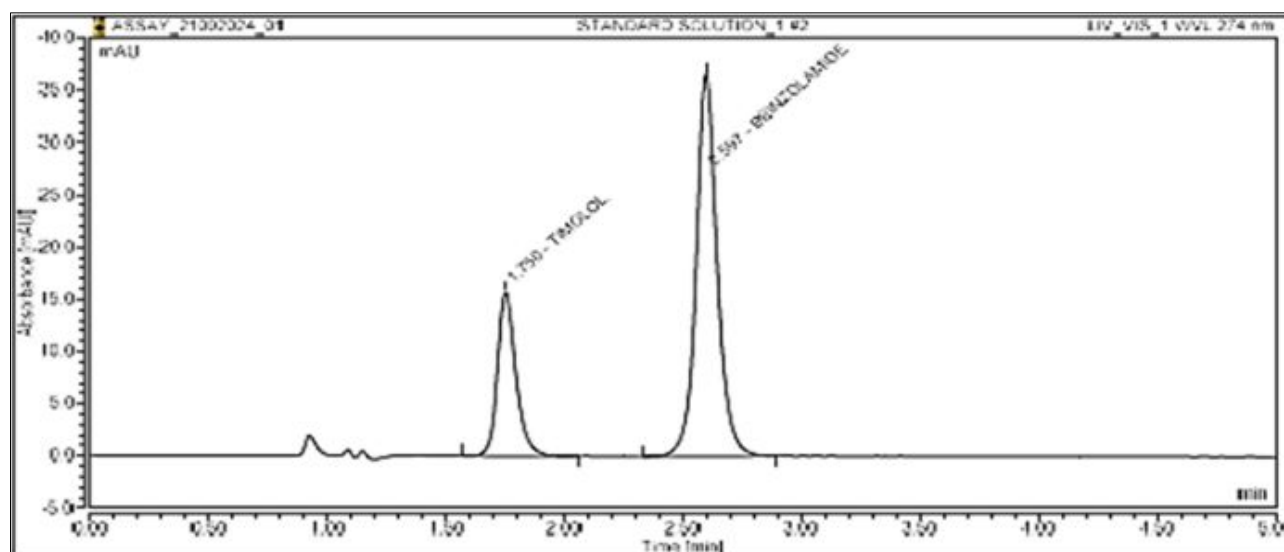
Accuracy Level	Concentration of standard added ($\mu\text{g}/\text{mL}$)	Concentration of standard recovered ($\mu\text{g}/\text{mL}$)	% Recovery	Average	% RSD
80%- 1	24.7900	24.6887	100	101	1.68
80%- 2	24.7900	25.3733	102		
80%- 3	24.7900	25.2644	102		
100%-1	49.5800	48.0359	98	99	0.52
100%-2	49.5800	49.1823	99		
100%-3	49.5800	48.2554	98		
120%-1	74.3700	72.9176	98	99	0.83
120%-2	74.3700	73.9057	99		
120%-3	74.3700	73.0231	98		

Precision: The percentage RSD of system precision observed for standard solution area was 0.1 and 0.2 for Timolol and Brinzolamide peaks which was well below the 2.0% threshold. The method precision was studied with six different sample preparations and % RSD value reported for assay was 0.1 and 0.2 for Timolol and Brinzolamide content, which was within the 2 percent boundaries of the RSD limit range. Similarly intermediate precision also

performed on six sample preparation and results 0.1% and 0.2% also fell within the 2 percent boundaries of the RSD limit range. Both the absolute difference between the precision of the methods and the intermediate precision did correlate and were within the given limits. Consequently, Tables 5 and 6 showed that the approach was accurate in terms of system precision, method precision, and intermediate precision of the method.

Table 5: System precision

Parameter	Timolol	Brinzolamide	Acceptance Limit
Tailing factor	1.25	1.08	NMT 2.0
Theoretical plates	2623	4506	NLT 1000
% Relative standard deviation (RSD)	0.80	0.62	NMT 2.0%
Retention time (in minutes)	1.75	2.59	-

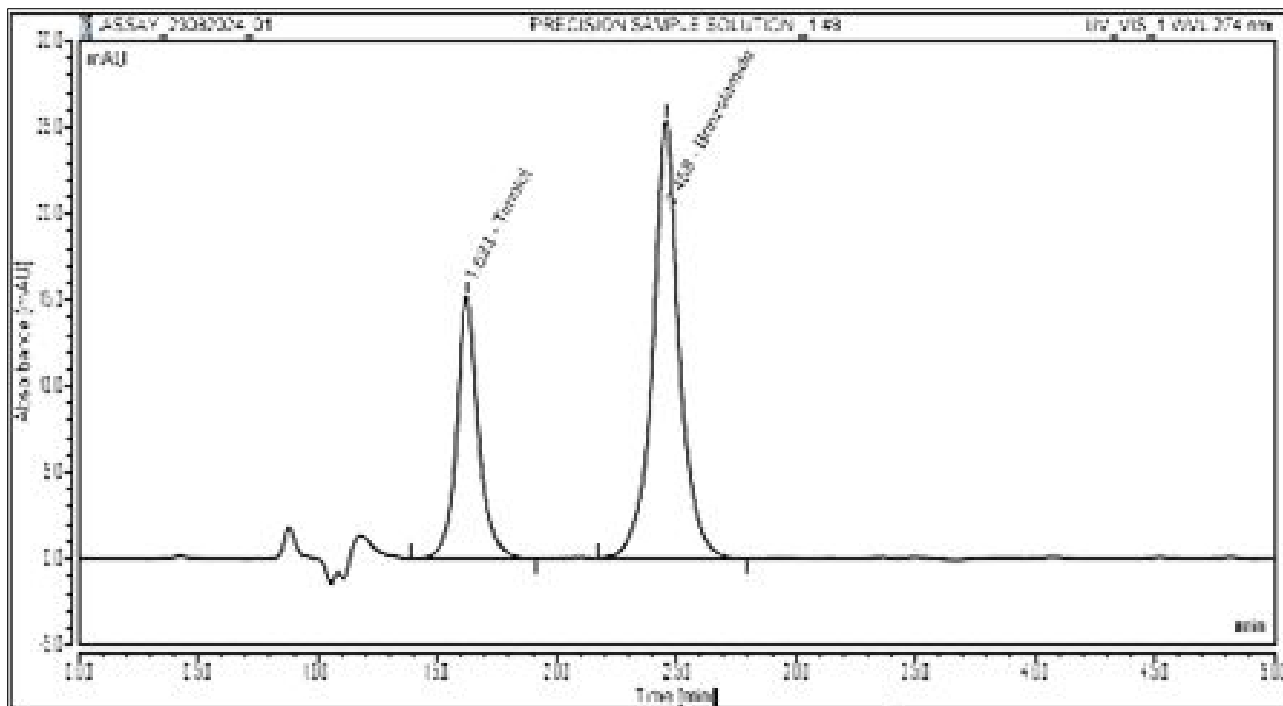


Peak No.	Peak Name	Ret. Time min	Area mAU*sec	RRT	Asymmetry (EP/USP)	Plates (USP)	Resolution (USP)
1	TIMOLOL	1.750	84.867	1.00	1.25	2623	n.a.
2	BRINZOLAMIDE	2.597	225.578	1.48	1.08	4506	5.81

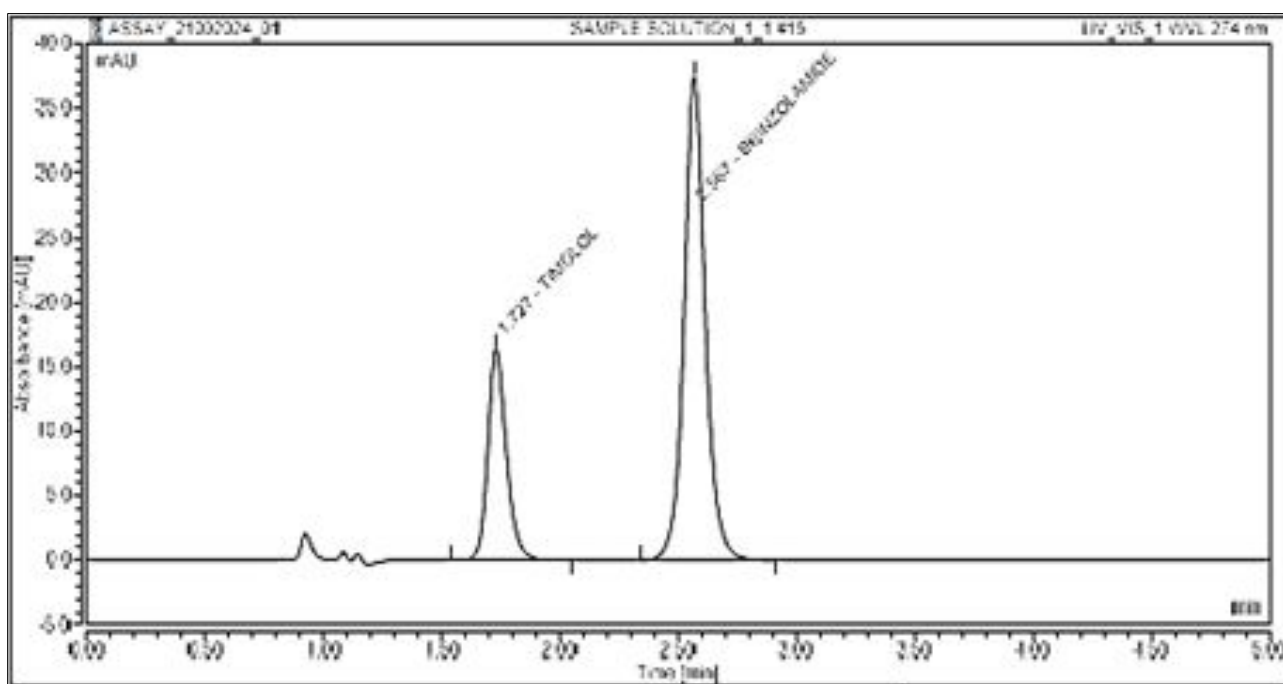
Fig 7: System precision Chromatograph

Table 6: Evaluation of Intermediate and method precision

Sr. No.	Repeatability		Intermediate precision	
	Timolol	Brinzolamide	Timolol	Brinzolamide
1	98.6	101.1	100.2	99.4
2	98.6	102.1	100.6	101.2
3	98.2	102.9	100.0	100.7
4	98.6	101.5	100.6	99.9
5	99.8	103.0	100.1	99.5
6	100.5	101.4	100.7	100.2
Mean	99.1	102.0	100.4	100.2
S. Dev.	0.8939	0.8050	0.3011	0.7007
% RSD	0.90	0.79	0.30	0.70



a. Method precision Chromatograph



b. Intermediate precision Chromatograph

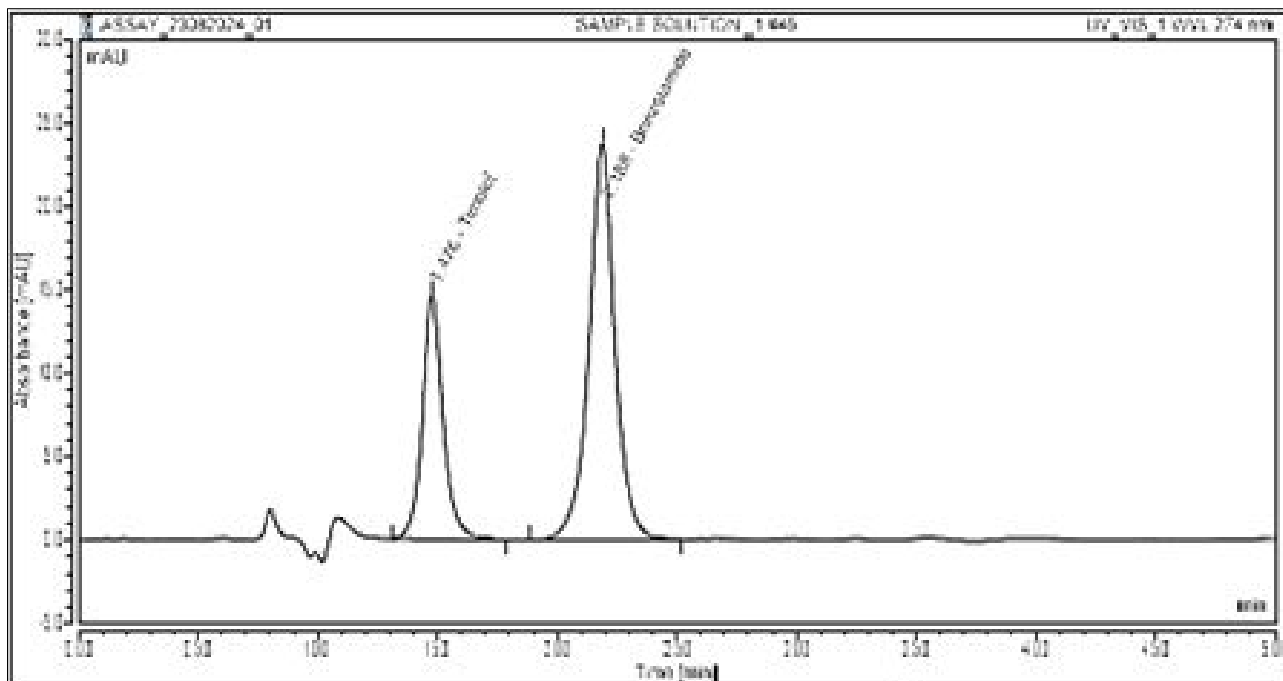
Fig 8: Method precision/ Intermediate precision chromatograph of test

Robustness: The robustness parameters such as column oven temperature ($\pm 5^\circ\text{C}$), flow rate ($\pm 0.1\text{mL}$), mobile phase buffer pH (± 0.2) were performed and their percentage RSD was calculated (Table 7) which was found

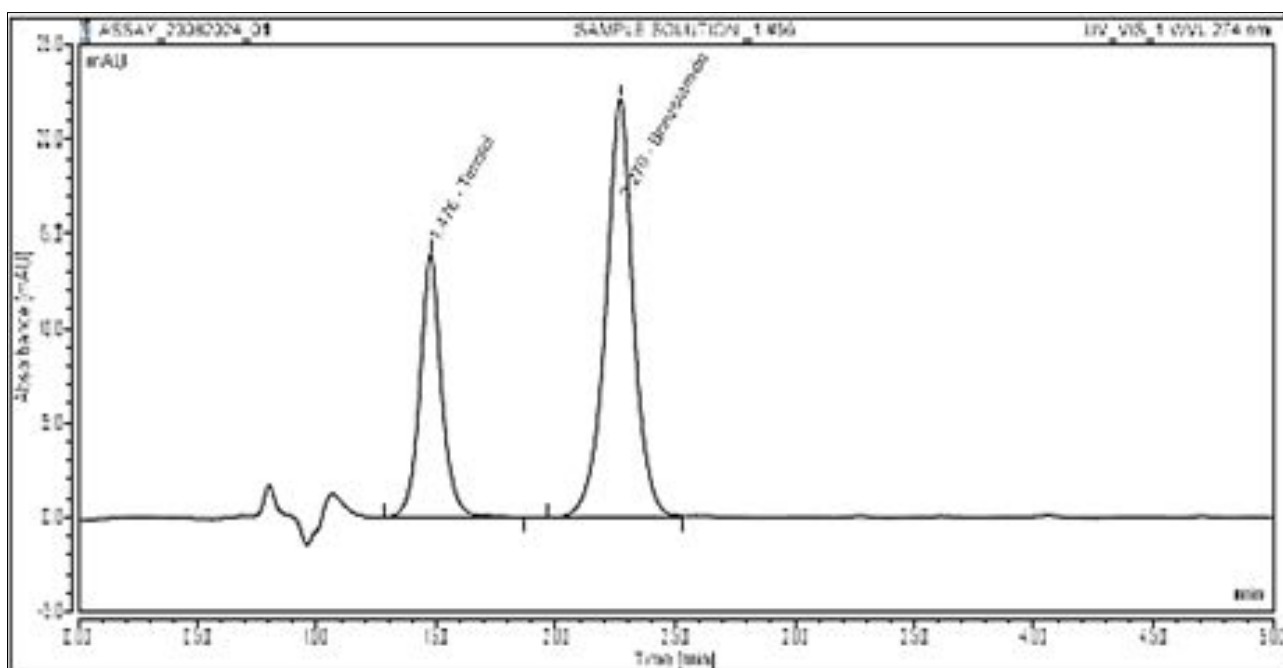
within the limit. No significant changes were observed in the results even after changing the above parameters and hence we have concluded that the above method is robust as per ICH validation guideline.

Table 7: Evaluation of Robustness Parameter

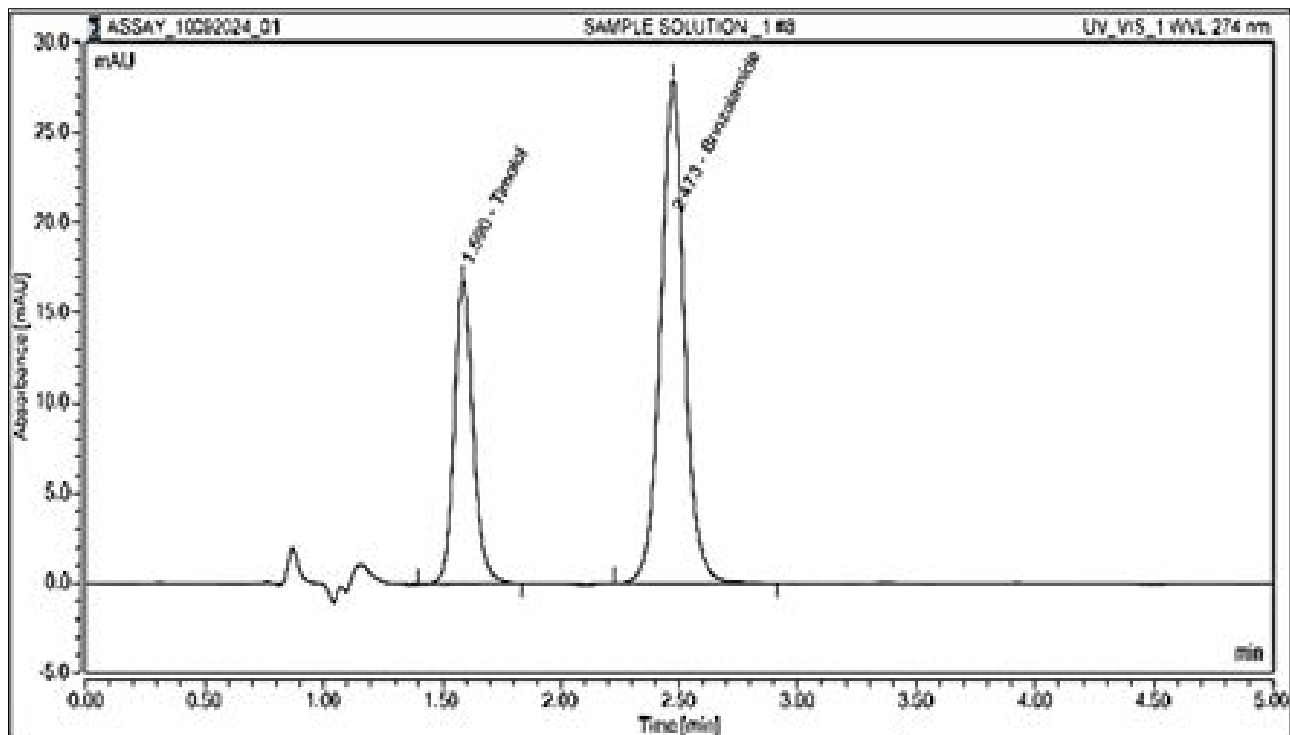
Sr. No.	Parameter change	Absolute Difference	Timolol	Brinzolamide
1	Column Temperature 40°C	2.0%	98.8	101.9
2	Column Temperature 50°C		99.6	101.2
3	Buffer pH 4.15		98.5	102.1
4	Buffer pH 4.35		100.4	102.7
5	Flow Rate (0.9 mL/ Min)		100.0	102.4
6	Flow Rate (1.1 mL/ Min)		100.5	102.1



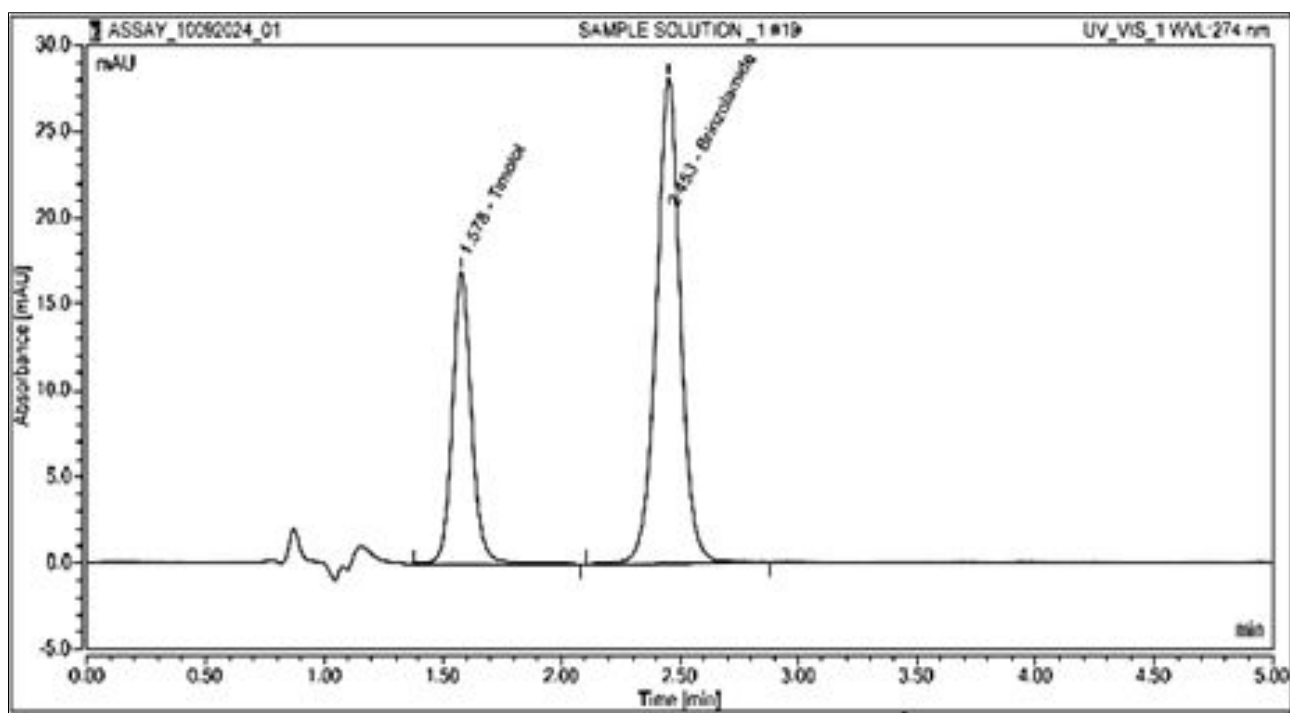
a. Column Temp 40 °C



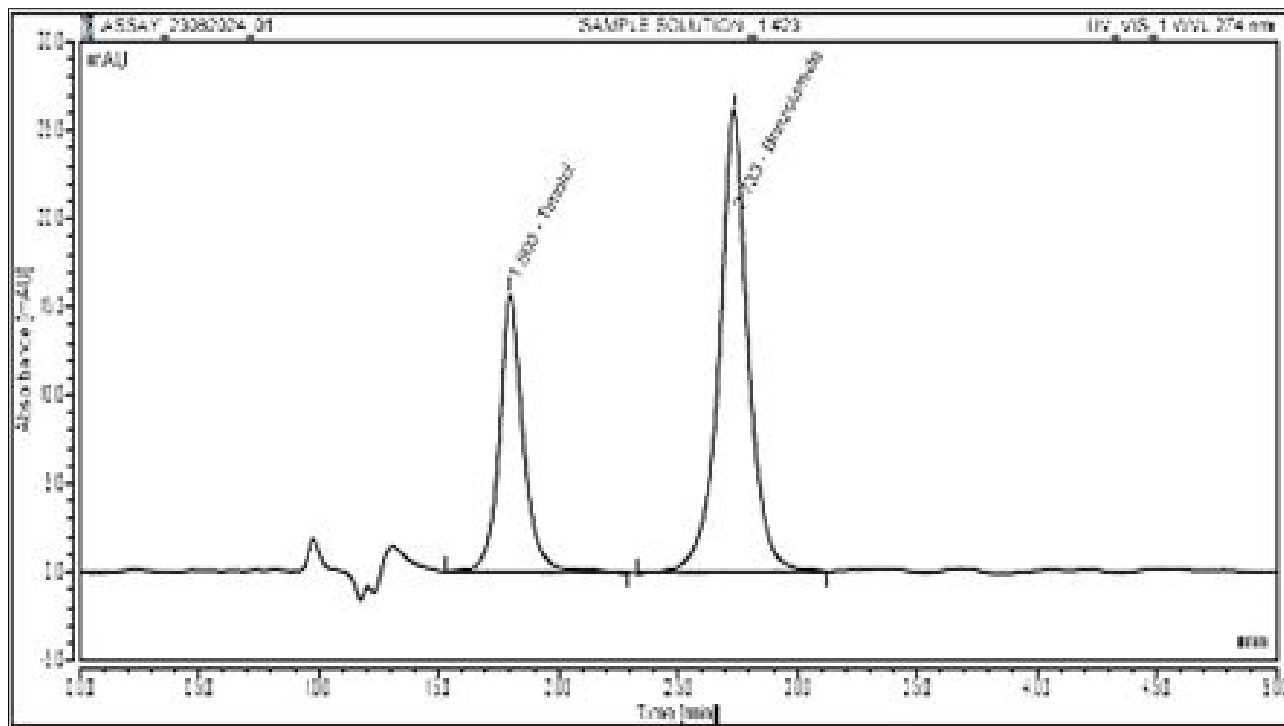
b. Column Temp 50 °C



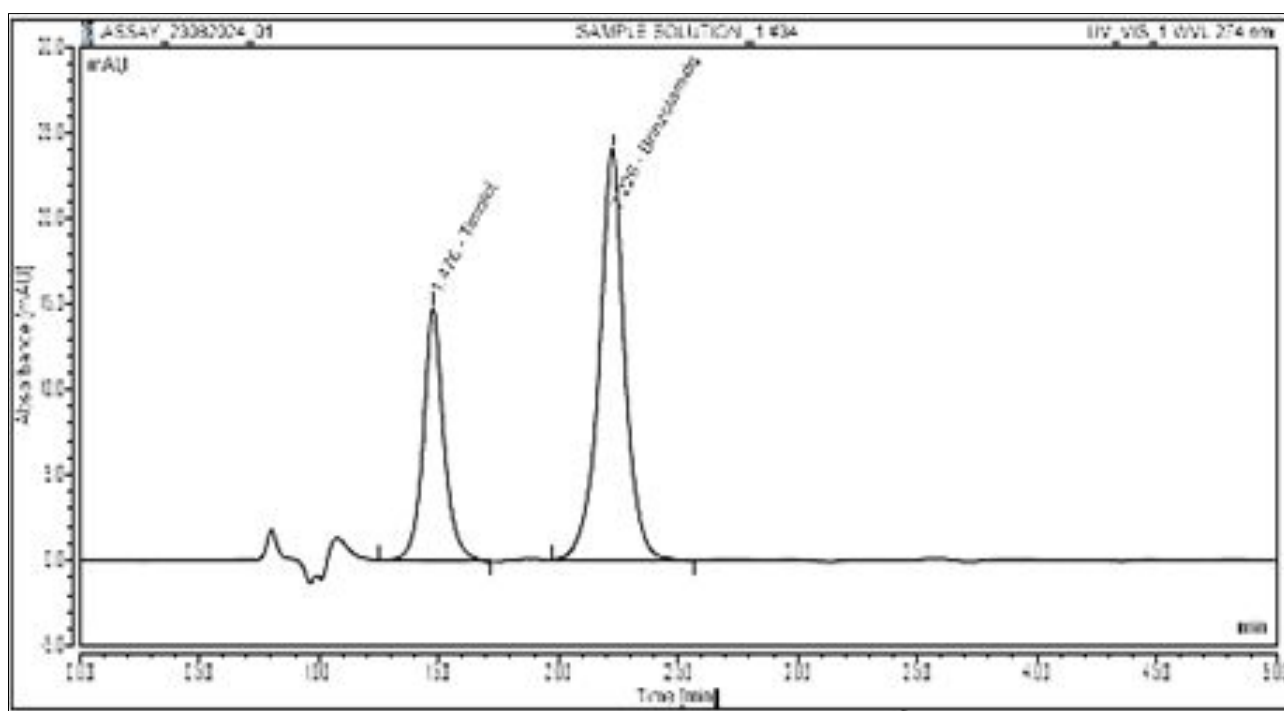
c. Buffer pH 4.05



d. Buffer pH 4.45



e. Flow Rate (0.9 mL/ Min)



f. Flow Rate (1.1 mL/ Min)

Fig 9: Robustness parameter chromatograph of test**Degradation study****Neutral degradation study**

The stress level of the test sample was checked by refluxing the test sample quantity at 60 °C for 6 hours under neutral conditions. The resulting solution was diluted up to the mark and injected in HPLC system in order to check the stability of the sample.

Thermal degradation study

The test sample was kept in the oven at 105°C for 6 hours for thermal degradation studies. Subsequently after cooling

to room temperature the test sample was diluted up to the mark to obtain the desired concentration and injected in the HPLC device in order to understand the thermal stability of the sample.

Photolytic stability study

The photolytic stability of the test sample was studied by irradiating the test sample to UV-visible light by keeping the sample in a glass beaker in UV photo chamber for 1.2 million Lux hours or 200 watt hours/m². The resulting solution was diluted up to the mark to get the desired

concentration and then inserted into the HPLC chromatographic system.

Acid degradation study

Acidic stress level of the sample was analyzed by addition of 1 mL of 2N hydrochloric acid into the sample and refluxing at 60 °C for 30 minutes. Subsequently the resulting solution was cooled and neutralized with 1 mL of 2N sodium hydroxide solution and diluted up to the mark to obtain the desired concentration. HPLC chromatographic system was used to evaluate its acidic stability.

Base degradation study

Alkali stress degradation of the sample was analyzed by addition of 1 mL of 2N sodium hydroxide into the sample and refluxing for 30 minutes at 60 °C. Subsequently the

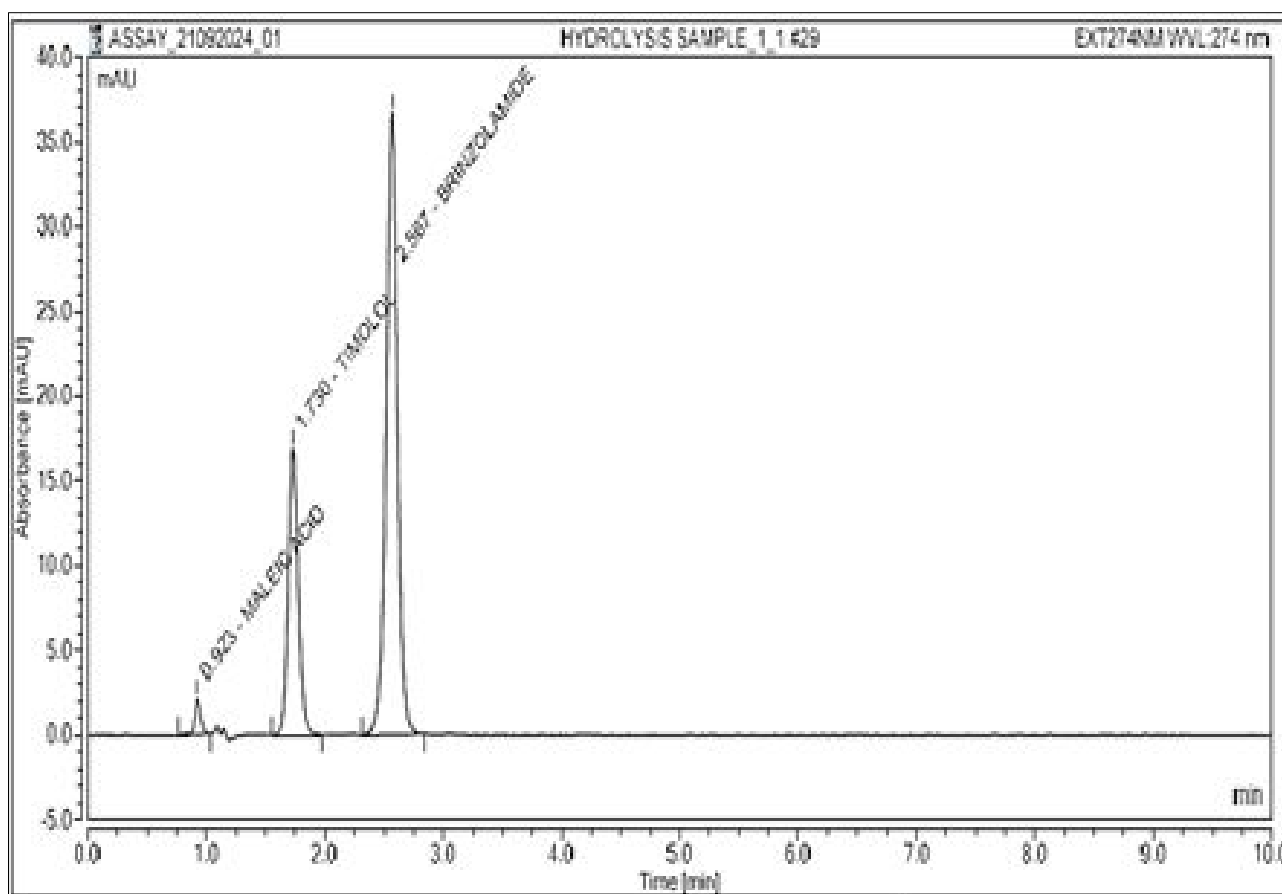
resulting solution was cooled and neutralized with 1 mL of 2N hydrochloric acid solution. Further dilution of the resulting solution was done in order to obtain the desired concentration. The resulting solution was injected in the HPLC system to evaluate its alkali stability.

Peroxide degradation study

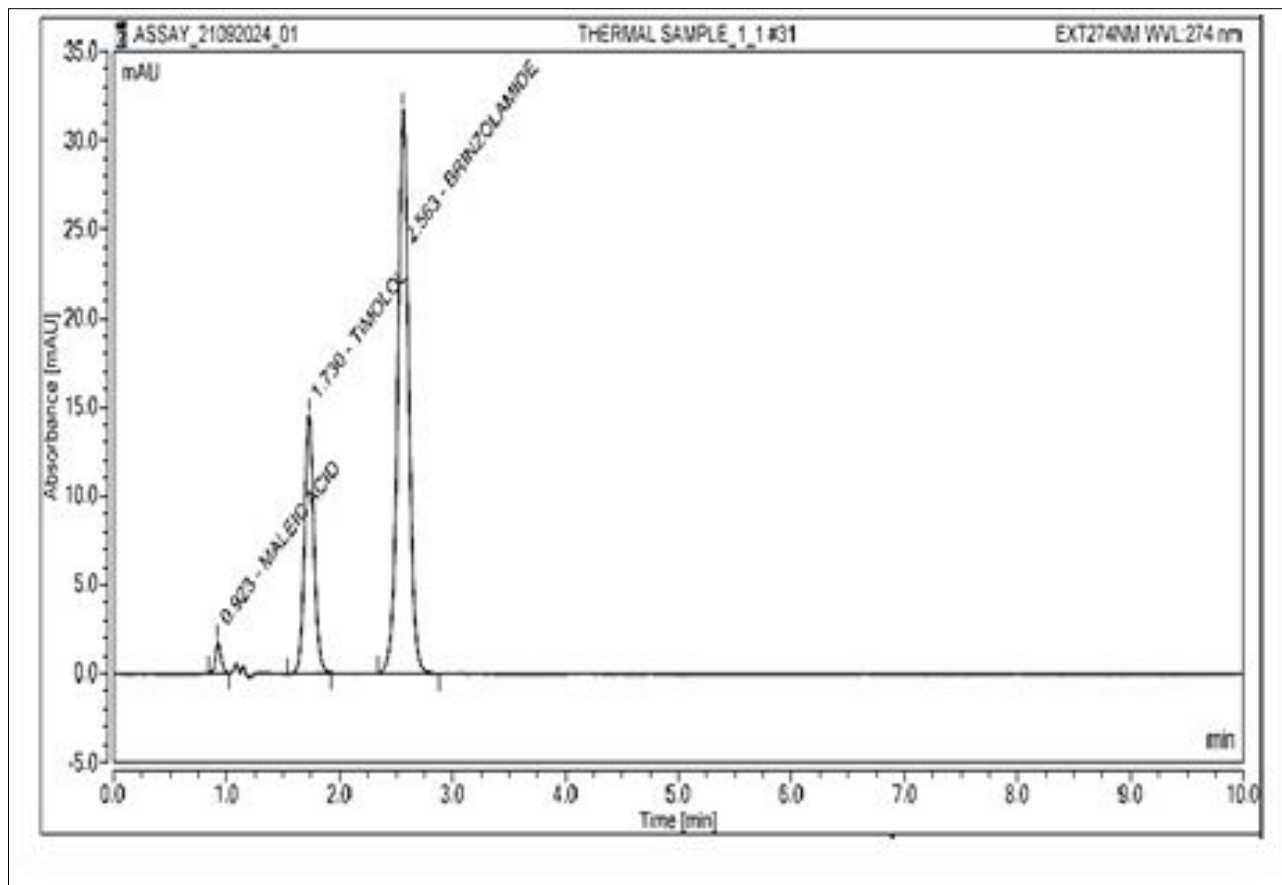
Oxidative degradation of sample was evaluated by addition of 1 mL of 3% hydrogen peroxide solution into the sample and refluxing for 30 minutes at 60 °C. After cooling the resulting solution 1 mL of reducing agent sodium metabisulfate solution was added and diluted up to the mark to obtain the desired concentration. The resulting solution was injected in the HPLC device for peroxide degradation study. The results are shown in Table 8.

Table 8: Evaluation of Forced degradation

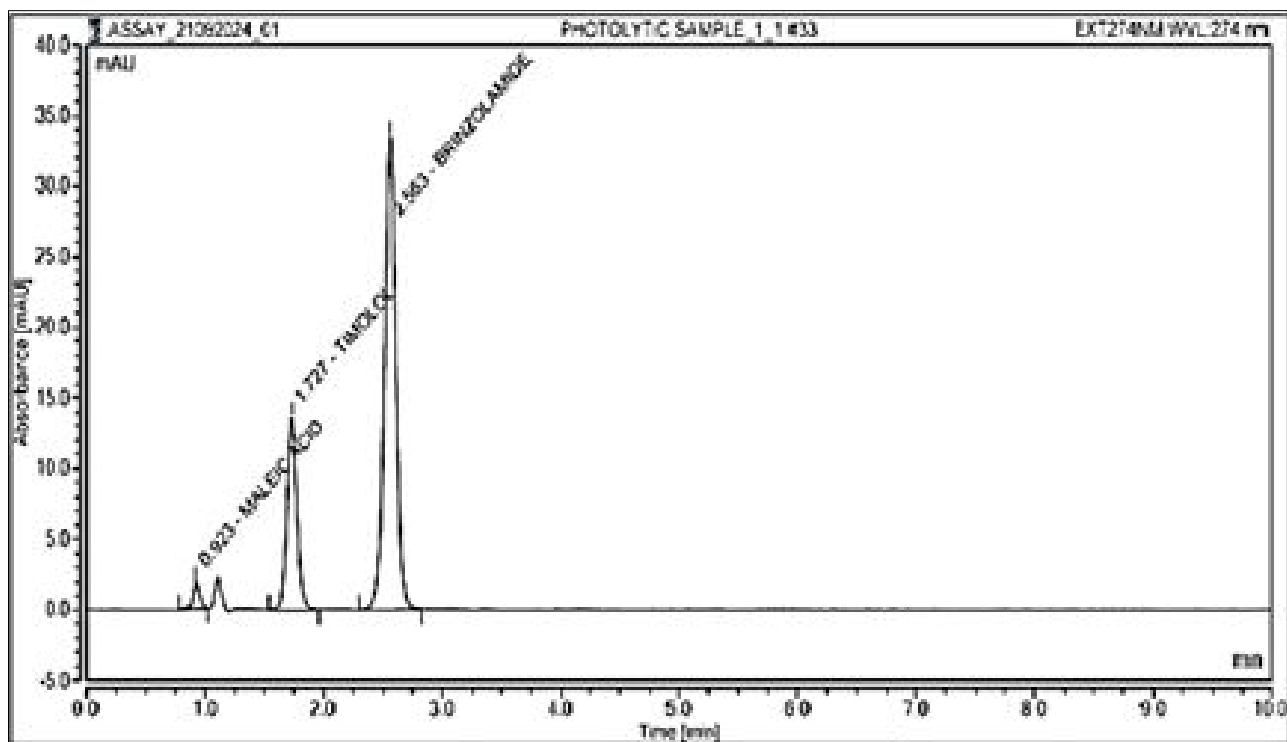
Sr. No.	Type of degradation	Timolol			Brinzolamide		
		% Assay	% Release w.r.t digit	% Deg.	% Assay	% Release w.r.t digit	% Deg.
1	Neutral	102.5	100.0	0.0	100.6	100.0	0.0
2	Thermal	102.5	100.0	0.0	100.4	99.8	0.2
3	Photolytic	91.4	89.2	10.8	101.1	100.5	0.5
4	Acid	102.9	100.3	0.3	98.9	98.3	1.7
5	Base	78.1	76.2	23.8	84.7	84.2	15.8
6	Oxidative	31.3	30.5	69.5	80.4	79.9	20.1



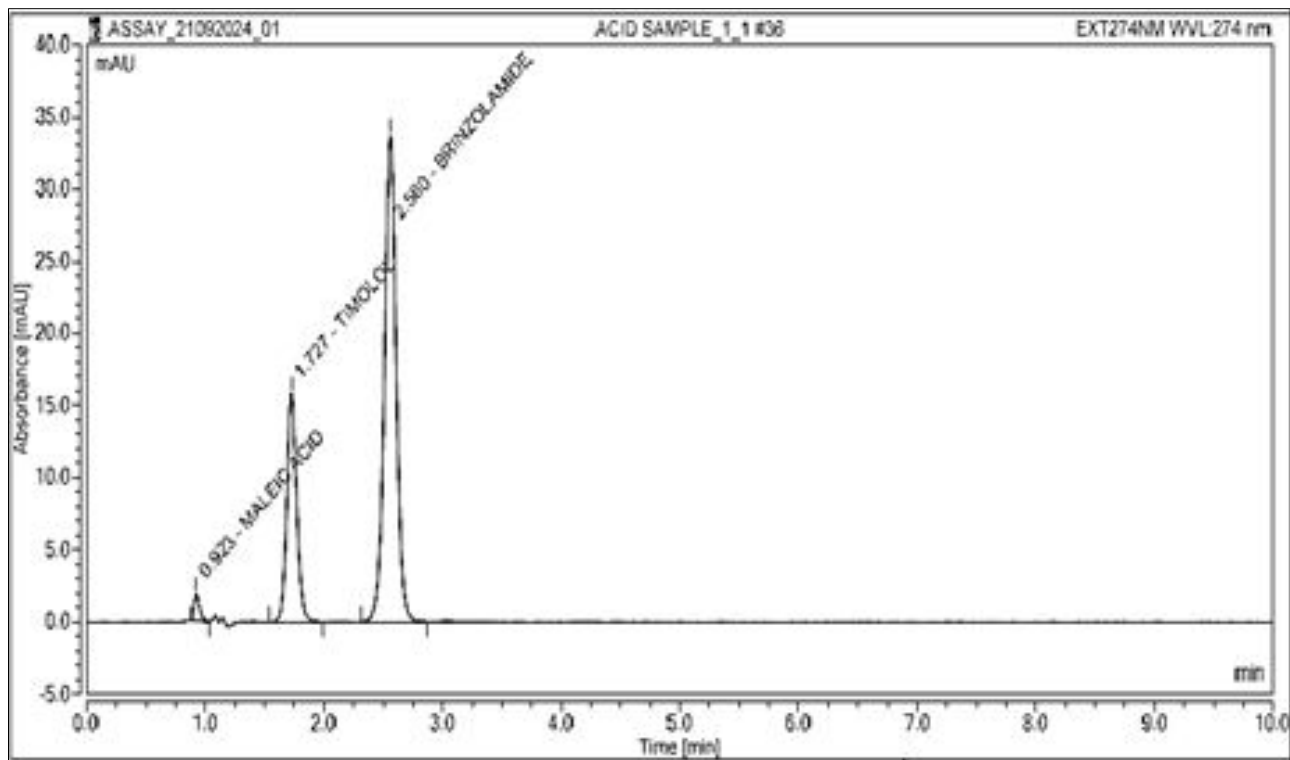
a. Neutral Condition Chromatograph



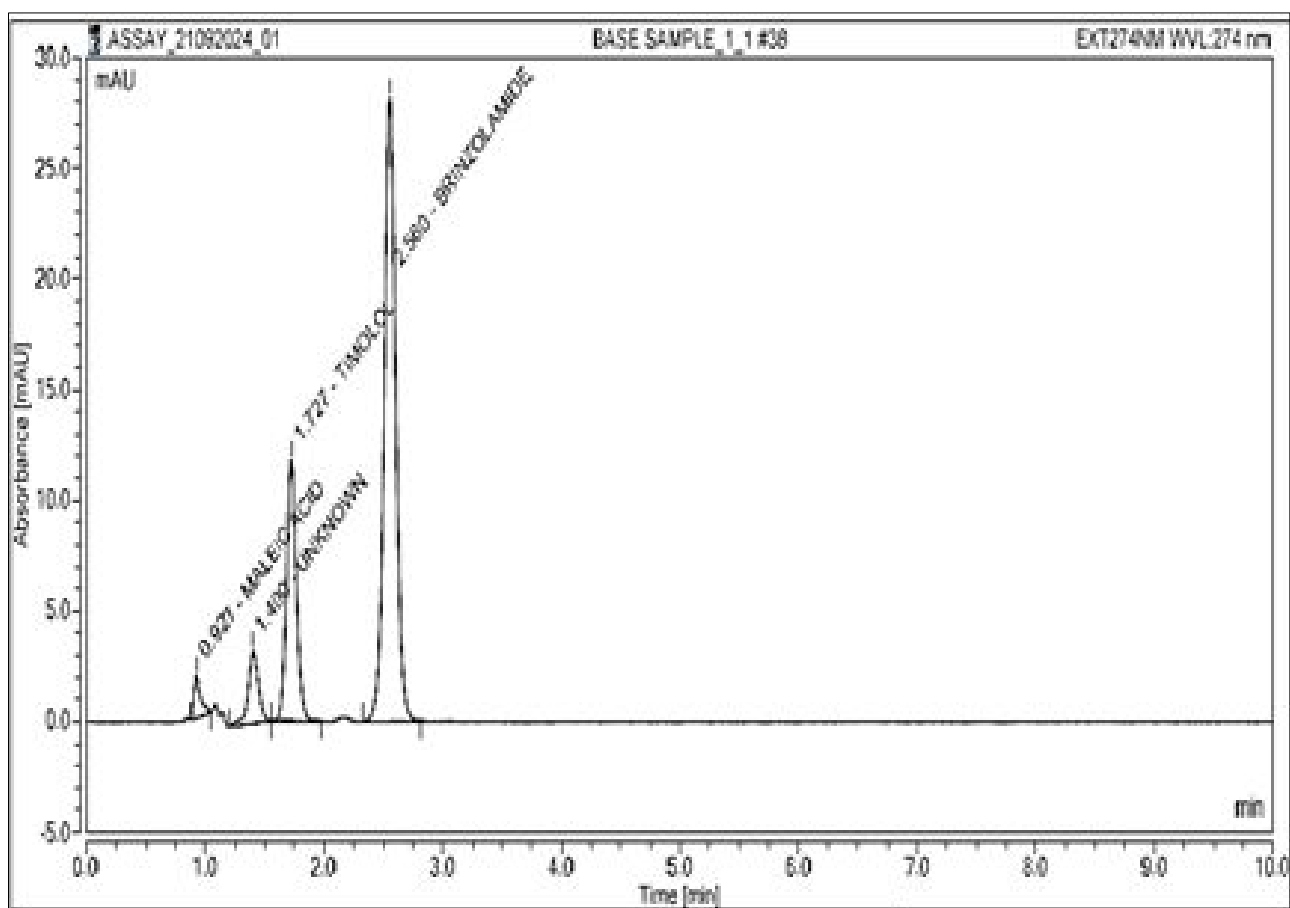
b. Thermal Condition Chromatograph



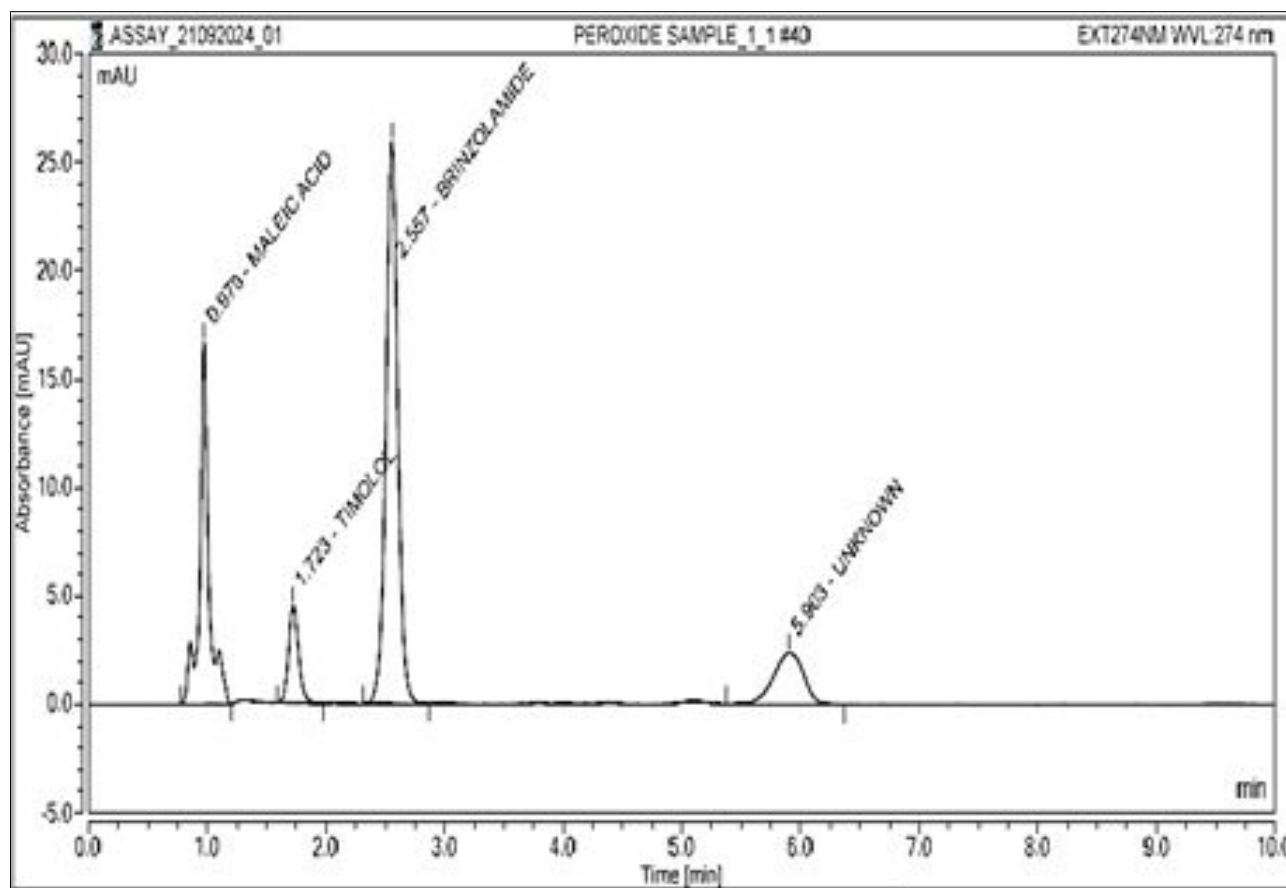
c. Photolytic Condition Chromatograph



d. Acid degradation Chromatograph



e. Base degradation Chromatograph



f. Oxidative degradation

Fig 10: Forced degradation of TIM and BRZ test solution**Conclusion**

The analytical method developed for Timolol and Brinzolamide quantification in this work, was validated successfully as per ICH guideline Q2(R2) and USP <1225>. This method was found to be specific, precise, linear, accurate and robust. This method will be beneficial for qualitative as well quantitative determination of Timolol and Brinzolamide used in combination in process and final formulation.

Acknowledgement

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Conflict of interest

The authors disclose that there is no conflict of interest.

Abbreviations

- **ICH:** International conference of Harmonization
- **RP-HPLC:** Reverse Phase- High Performance Liquid Chromatography
- **RSD:** Relative Standard Deviation
- **QC:** Quality Control
- **NLT:** Not Less Than
- **NMT:** Not More Than
- **TIM:** Timolol
- **BRZ:** Brinzolamide

Summary

- A new short and precise, robust method has been evolved for assessment of Timolol Maleate and Brinzolamide in their ophthalmic dosage form.
- A new isocratic analytical technique has been introduced for the identification and quantification of both the active components in a short period time.
- The retention time of Timolol and Brinzolamide has been detected as 1.7 minutes and 2.5 minutes with good resolution.
- The estimation of Timolol and Brinzolamide has been validated as per USP <1225> and ICH guideline Q2(R2)
- All validation parameters were observed to be within the acceptable limits and hence this systematic stability indicating method can be used for qualitative as well quantitative purpose.

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