



Validated Chromatographic Method for Simultaneous Determination of Timolol Maleate and Dorzolamide Hydrochloride

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ABSTRACT:

A simple, precise, and robust analytical method was developed and validated for the simultaneous estimation of Timolol Maleate and Dorzolamide Hydrochloride using UV spectrophotometry and HPLC techniques. The absorption maxima were determined at 295 nm for Timolol Maleate and 254 nm for Dorzolamide Hydrochloride in orthophosphate buffer (pH 3.5). Calibration curves exhibited excellent linearity with correlation coefficients of 0.9956 (UV) and 0.9991–0.9992 (HPLC), confirming the reliability of the method. The HPLC analysis was performed using a Thermo P4000 system with column Chemsil ODS-C18 (250mm X 4.6 mm, 5 μ m) and PDA detector at 290 nm, employing a mobile phase of acetonitrile and phosphate buffer (60:40 v/v). Method validation parameters including accuracy, precision, specificity, robustness, LOD, and LOQ were assessed according to ICH guidelines. Recovery studies demonstrated high accuracy (99.2–100.0%), while precision results showed %RSD values below 2.0%, indicating reproducibility. The LOD and LOQ values were found to be 0.03 μ g/ml and 0.11 μ g/ml for Timolol Maleate, and 0.006 μ g/ml and 0.018 μ g/ml for Dorzolamide Hydrochloride, respectively. Robustness testing confirmed the method's reliability under small variations in flow rate and wavelength. System suitability parameters such as retention time, theoretical plates, and tailing factor were within acceptable limits. Overall, the developed method is specific, accurate, precise, and suitable for routine simultaneous quantification of Timolol Maleate and Dorzolamide Hydrochloride in pharmaceutical formulations.

INTRODUCTION:

The simultaneous estimation of multiple active pharmaceutical ingredients (APIs) in combined dosage forms is a critical aspect of modern pharmaceutical analysis. Fixed-dose combinations are widely prescribed to enhance therapeutic efficacy, improve patient compliance, and reduce dosing frequency^[1]. Among these, Timolol Maleate, a non-selective β -adrenergic receptor blocker, and Dorzolamide Hydrochloride, a carbonic anhydrase inhibitor, are commonly co-formulated in ophthalmic preparations for the treatment of glaucoma and ocular hypertension.

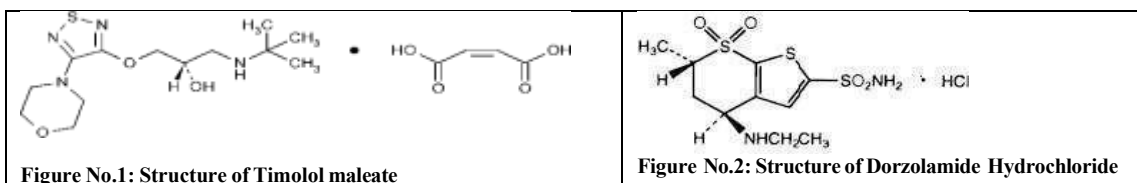
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Their combined action effectively reduces intraocular pressure by decreasing aqueous humor production through complementary mechanisms^[2]. Accurate and reliable quantification of these drugs in pharmaceutical formulations is essential to ensure product quality, safety, and efficacy^[3]. Traditional methods often focus on single-drug analysis, but simultaneous estimation requires robust analytical techniques capable of distinguishing and quantifying both compounds without interference. High-performance liquid chromatography (HPLC) and ultraviolet (UV) spectrophotometry are widely recognized for their sensitivity, reproducibility, and suitability in routine quality control laboratories^[4,5,6].

The present study focuses on the development and validation of a simple, precise, and accurate analytical method for the simultaneous estimation of Timolol Maleate and Dorzolamide Hydrochloride using UV spectrophotometry and HPLC. The method was validated according to ICH guidelines, assessing parameters such as linearity, accuracy, precision, robustness, specificity, limit of detection (LOD), and limit of quantification (LOQ)^[7,8]. By establishing a validated protocol, this work aims to provide a reliable analytical tool for routine pharmaceutical analysis of fixed-dose combinations containing Timolol Maleate and Dorzolamide Hydrochloride.



MATERIALS AND METHODS:

Reagents and sample:

All chemicals and reagents employed in this study were of analytical grade anhydrous disodium hydrogen phosphate, sodium hydroxide, HPLC grade water, orthophosphoric acid. HPLC grade Acetonitrile and water. Timolol Maleate and Dorzolamide Hydrochloride were obtained from cipla, Mumbai.

Instrumental and chromatographic condition:

UV spectrum was taken using Double beam UV-VIS spectrophotometer (UV-1700, Shimadzu, India). Chromatographic separation was carried out using a Thermo P4000 HPLC system equipped with column Chemsil ODS-C18 (250mm X 4.6 mm , 5 μ m) ,PDA detector and Chromquest software. The stationary phase was maintained at 25°C, and the injection volume was 20 μ L. Detection was performed at 290 nm, with a total run time of 15 minutes. The system was equilibrated prior to each injection to ensure reproducibility.

Standard calibration curve for Timolol Maleate and Dorzolamide Hydrochloride by UV:

Determination of absorption maxima:

A solution of Timolol Maleate and Dorzolamide Hydrochloride containing the concentration 10 μ g/ml was prepared in orthophosphate buffer 3.5 pH ; UV spectrum was taken using Double beam UV-VIS spectrophotometer (UV-1700, Shimadzu, India). The solution was scanned in the range of 200 – 400 nm.

Preparation calibration curve:

For Timolol Maleate: the Timolol Maleate drug 10 mg was weighed accurately and then it dissolve in the 10 ml orthophosphate buffer of pH 3.5 in the 10 ml of volumetric flask and Dorzolamide Hydrochloride: The Dorzolamide Hydrochloride drug 10 mg was weighed accurately and then it dissolve in the 10 ml orthophosphate buffer of pH 3.5 in the 10 ml of volumetric flask for the, then to make (1000 μ g/ml) standard stock solution. Then from it take 1 ml of stock solution was taken from the 10 ml volumetric flask, to make (100 μ g/ml) standard stock solution. 1.2 ml stock solution was taken in another 10 ml volumetric flask and then final concentrations were prepared ranged 5-10 μ g/ml with orthophosphate buffer 3.5 pH for Timolol Maleate and 10-50 μ g/ml ranged for Dorzolamide Hydrochloride. The absorbance of standard solution was determined using UV/ VIS spectrophotometer for Timolol Maleate and Dorzolamide Hydrochloride at 295 nm and 254 nm respectively.

Procedure for Preparation of mobile phase:

A mixture of Acetonitrile and ortho phosphate buffer (pH 3.5) in the ratio of 60:40 v/v was mix. Then the solution was filtered through 0.45 μ nylon membrane filter, degassed and used as the mobile phase.

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Procedure for Preparation of pH 3.5 buffer solution:

To prepare the buffer solution, 34.0 g of sodium dihydrogen phosphate (anhydrous) and 12 g of orthophosphoric acid were dissolved in five liters of water. The pH of this thoroughly mixed solution was adjusted (if found necessary) with 10 N NaOH so that the resultant pH was approximately 3.5 ± 0.05 . Before use, filtration of the mixture through 0.45 μ nylon membrane filter paper and degassing was carried out.

Preparation of standard stock solution:

Standard stock solution was prepared by dissolving accurately weighed 100 mg of Timolol Maleate and Dorzolamide Hydrochloride in mobile phase and the volume was made up to 100 ml with mobile phase in 100 ml volumetric flask (Stock solution-I, 1000 mcg / ml). 10 ml of stock solution-I was diluted to 100 ml with mobile phase (Stock solution-II, 100 mcg / ml). 1 ml of stock solution-II was taken in 10 ml standard flask diluted to 10 ml with mobile phase to get the concentration 10 mcg / ml. The absorbance of resulting solution was measured against respective blank solution in the UV region of 200-400 nm, which shows maximum absorbance of Timolol Maleate and Dorzolamide Hydrochloride at 290 nm.

Simultaneous Estimation of Timolol Maleate and Dorzolamide Hydrochloride:

1. Linearity:

Appropriate volume from the stock solution was diluted to get the final concentration of 5-10 μ g/mL and 10-50 μ g/mL for Timolol Maleate and Dorzolamide Hydrochloride respectively. Then the chromatogram were recorded, for each concentration, plotted the graph concentration versus number of theoretical plates. Each level solution was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

Accuracy:

Assay was performed in triplicate for various concentrations of Timolol Maleate and Dorzolamide Hydrochloride equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system per the test procedure.

Preparation of Standard stock solution:

Weigh accurately about 10 mg of Timolol Maleate and Dorzolamide Hydrochloride and transferred into a 100ml clean dry volumetric flask about 70ml of diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same solvent (Stock solution). Further 1.2 and 5 ml of Timolol Maleate and Dorzolamide Hydrochloride from above stock solutions were pipette into a 10ml volumetric flask and diluted up to the mark with diluent. Chromatogram were shown in results.

Standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected in to HPLC system. Amount found and amount added for Timolol Maleate and Dorzolamide Hydrochloride individual recovery and mean recovery values were also calculated. The average % recovery of Timolol Maleate and Dorzolamide Hydrochloride were calculated

Precision:

A) Repeatability:

The standard solution was injected for five times and the area was measured for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. Relative standard deviation of % Assay results should not be more than 2.0.

B) Intermediate Precision (analyst to analyst variability):

To evaluate the intermediate precision (also known as the ruggedness) of the method precision was performed on different days by using different columns of same dimensions.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

The LOD and LOQ of the developed method were determined by analysing progressively low concentration of the standard solution using the developed methods. The LOD is the concentration of the analyte that gives a measurable response (signal to noise ratio 3.3). The LOQ is the lowest concentration of the analyte, which gives

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a response that can be accurately quantified (signal to noise ratio of 10).

Robustness:

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and different wavelength, temperature variations which may differ but the responses were still within the specified limits of the assay.

A) Effect of variation of flow rate:

A study was conducted to determine the effect of variation in flow rate. The flow rate was varied at 0.8ml/min to 1.2 ml/min. Standard solution 100 ppm of Timolol Maleate and Dorzolamide Hydrochloride were prepared and analyzed using the varied flow rates along with method flow rate. The results are summarized. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate $\pm 10\%$. The method is robust only in less flow condition. The effect of variation of flow rate was evaluated.

b) Effect of variation of Wavelength:

A study was conducted to determine the effect of variation in wavelength. The Wavelength was varied from ± 2 nm. Standard solution 100 $\mu\text{g/ml}$ of Timolol Maleate and Dorzolamide Hydrochloride were prepared and analyzed using the varied wavelength along with the actual wavelength in the method. Standard solution was prepared and injected into the HPLC system. The retention time values were measured.

System Suitability:

Sample solution of Timolol Maleate and Dorzolamide Hydrochloride were injected three times into HPLC system as per test procedure. The system suitability parameters were evaluated from standard chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from three replicate injections.

RESULTS AND DISCUSSION:

Analytical method development:

Determination of Working Wavelength (λ_{max}) by UV:

10 gm of the Timolol Maleate and Dorzolamide Hydrochloride standard drug is taken in a 10 ml volumetric flask and dissolved in orthophosphate buffer of pH 3.5 and volume made up to the mark, from this solution. 0.1ml is pipetted into 10ml volumetric flask and made upto the mark with the orthophosphate buffer of pH 3.5 to give a concentration of 10 $\mu\text{g/ml}$. The above prepared solution is scanned in UV between 200-400 nm using water as blank. The λ_{max} was found to be 295 and 254 nm.

Calibration curve of drug Timolol Maleate:

Table No.1: Absorbance of Timolol Maleate by UV

Sr. No.	Conc.($\mu\text{g/ml}$)	Absorbance
1	0	0.00
2	5	0.124
3	10	0.302
4	15	0.410
5	20	0.583
6	25	0.759
	Scanning range	200-400nm
	Solvent	orthophosphate buffer of pH 3.5
	Amax	295 nm

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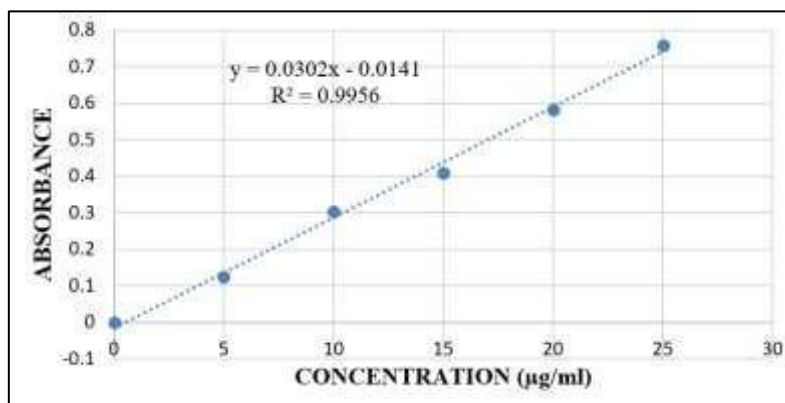


Figure No.3: Calibration Curve of Drug Timolol Maleate

The UV absorbance data obtained for drug Timolol Maleate (as shown in table) in solvent showed that absorbance of dilute drug solutions was progressively increased as increase in conc. The regression coefficient (R^2) value 0.9956 of Timolol Maleate obtained from graph (figure), confirmed the linear relationship between the variables (i.e., conc. and absorbance respectively) investigated.

Determination of λ max of drug Dorzolamide Hydrochloride by UV:

The λ max of drug was determined by UV spectroscopy using orthophosphate buffer of pH 3.5 as described in methodology section. The spectrum of the drug is presented in figure. The maximum absorption of drug Dorzolamide Hydrochloride in orthophosphate buffer of pH 3.5 was observed at 254 nm and therefore, this absorbance was selected as their λ max and used throughout the study for the analysis of drug.

Calibration curve of drug Dorzolamide Hydrochloride

Table No.2: Absorbance of Dorzolamide Hydrochloride by UV

Sr. No.	Conc.(µg/ ml)	Absorbance
1	0	0.00
2	10	0.145
3	20	0.289
4	30	0.367
5	40	0.480
6	50	0.623
Scanning range		200-400nm
Solvent		orthophosphate buffer of pH 3.5
λ max		254 nm

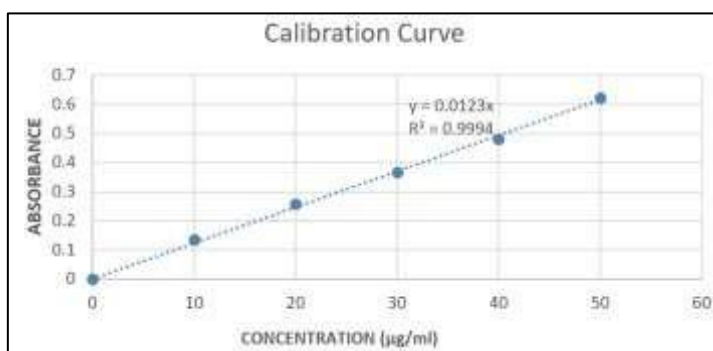


Figure No.4: Calibration Curve of Drug Dorzolamide Hydrochloride

The UV absorbance data obtained for drug Dorzolamide Hydrochloride (as shown in table) in orthophosphate buffer of pH 3.5 showed that absorbance of dilute drug solutions was progressively increased as increase in conc. The regression coefficient (R^2) value 0.998 of Dorzolamide Hydrochloride obtained from graph (figure), confirmed the linear relationship between the variables (i.e., conc. and absorbance respectively) investigated.

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Method Validation for the simultaneous estimation of Timolol Maleate and Dorzolamide Hydrochloride:

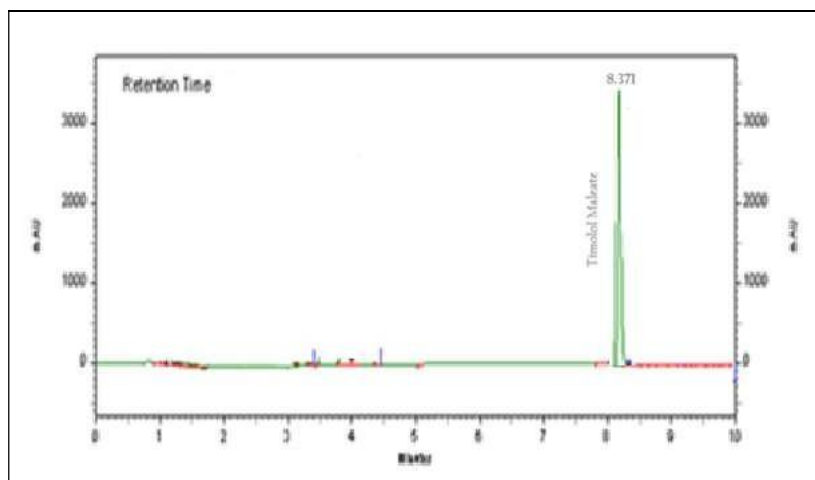


Figure No.5: Standard Chromatogram of Timolol Maleate

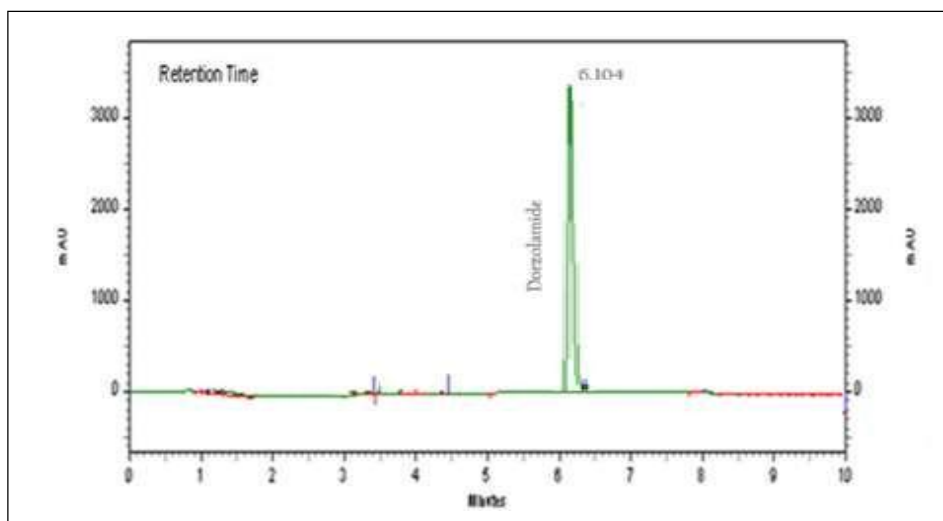


Figure No.6: Standard Chromatogram of Dorzolamide Hydrochloride

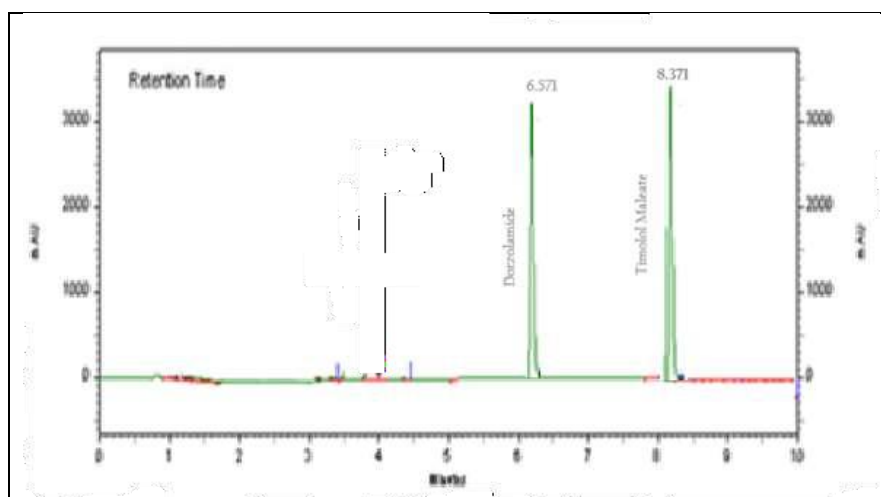


Figure No.7: Chromatogram of Timolol Maleate and Dorzolamide Hydrochloride at 290 nm
Linearity

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Linearity was studied by analysing five standard solutions covering the range of 5-25 µg/ml for Timolol Maleate and 10-50 µg/ml and Dorzolamide Hydrochloride. From the primary stock solution 0.5ml, 1ml, 1.5 ml, 2 ml, 2.5 ml of aliquots are pipetted into 10 ml volumetric flasks and made up to the mark with the diluent to give a concentrations of 5 µg/mL, 10µg/mL, 15 µg/mL, 20 µg/mL and 25 µg/mL of Timolol Maleate and 10 µg/mL, 20µg/mL, 30µg/mL, 40µg/mL and 50 µg/mL Dorzolamide Hydrochloride. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions, and the obtained data were subjected to regression analysis using the least squares method.

Table No.3: Linearity data of Timolol Maleate and Dorzolamide Hydrochloride

Sr No.	Timolol Maleate		Dorzolamide Hydrochloride	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
1	5	1094892	10	791516
2	10	1575824	20	1146503
3	15	1938292	30	1473225
4	20	2372105	40	1821944
5	25	2847388	50	2233793

A linear relationship between peak areas versus concentrations was observed for Timolol Maleate and Dorzolamide Hydrochloride in the range of nominal concentration. Correlation coefficient was 0.9991 and 0.9992 for both Timolol Maleate and Dorzolamide Hydrochloride which prove that the method was found to be linear.

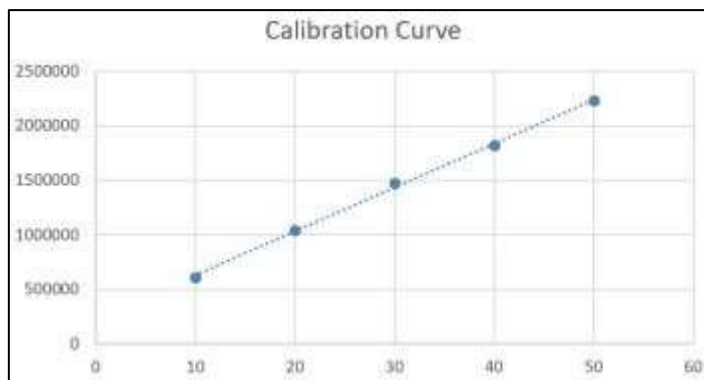


Figure No.8: Calibration Curve of Timolol Maleate by HPLC

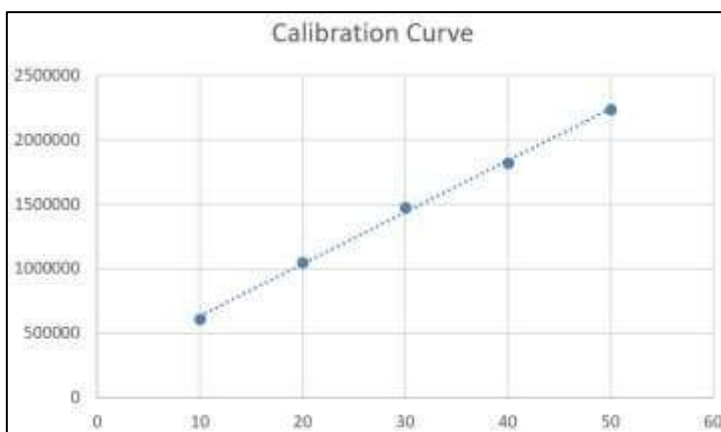


Figure No.9: Calibration curve of Dorzolamide Hydrochloride by HPLC

Method precision (Repeatability)

The precision of the method was checked by repeated preparation (n=6) of 10 µg/ml of Timolol Maleate and Dorzolamide Hydrochloride without changing the parameter of the proposed chromatographic method and measured the peak areas and retention time.

Table No. 4 : Summary of peak areas for method precision of Timolol Maleate and Dorzolamide Hydrochloride

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Sample No	Timolol Maleate			Dorzolamide Hydrochloride		
	Retention time (min)	Peak area	% Assay	Retention time(min)	Peak area	% Assay
1	8.372	1926157	100.1	6.572	1433404	100.2
2	8.371	1942639	99.5	6.571	1438312	99.7
3	8.371	1932993	99.6	6.571	1436765	99.4
4	8.370	1890193	98.6	6.572	1393554	99.2
5	8.371	1933047	98.8	6.573	1439888	99.6
6	8.370	1956298	99.4	6.574	1453754	99.4
Mean	8.371	1930221	99.4	6.572	1432613	99.6
%RSD	0.09	1.15	0.55	0.018	1.42	0.35

Results of variability were summarized in the above Table. Percentage relative standard deviation (%RSD) was found to be less than 2.0% which proves that method is precise.

Accuracy (% Recovery)

The accuracy of the method was determined by calculating the recoveries of Timolol Maleate and Dorzolamide Hydrochloride by analysing solutions containing approximately 50%, 100% and 150% of the working strength of Timolol Maleate and Dorzolamide Hydrochloride, the percentage recovery results obtained were listed in Table

Table No.5: Recovery data for Timolol Maleate and Dorzolamide Hydrochloride

Sr. No.	Accuracy level	Injection	Timolol Maleate		Dorzolamide Hydrochloride	
			% Recovery	Average	% Recovery	Average
1	50%	1	99.5	99.4	99.1	99.2
		2	99.3		98.9	
		3	99.4		99.6	
2	100%	1	100.1	99.7	100.2	99.76
		2	99.5		99.7	
		3	99.6		99.4	
3	150%	1	99.3	99.8	100.0	100.0
		2	100.1		100.2	
		3	100		99.9	

Results of accuracy study are presented in the above Table. All the results indicate that the method is highly accurate.

Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively.

Table No. 6 : LOD and LOQ values Calculated from calibration curve

	Timolol Maleate (µg)	Dorzolamide Hydrochloride (µg)
LOD	0.03	0.006
LOQ	0.11	0.018

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and mobile phase composition on assay of the analyte of interest. Here the mobile phase varied ± 2 nm and flow rate was varied ± 0.2 ml/min. The results were shown in Table 7.

Table No.7: Results of Robustness data for Timolol Maleate and Dorzolamide Hydrochloride

Parameter	RT		Theoretical plates		Asymmetry	
	Timolol Maleate	Dorzolamide Hydrochloride	Timolol Maleate	Dorzolamide Hydrochloride	Timolol Maleate	Dorzolamide Hydrochloride
Decreased flow rate (0.8ml/min)	8.697	6.210	4947	10078	1.17	1.22
Increased flow rate (1.2ml/min)	8.115	6.074	4364	8880	1.14	1.17
Wavelength 288 nm	8.372	6.572	4722	9498	1.18	1.20
292 nm	8.371	6.571	4664	9456	1.17	1.20

The results of Robustness of the present method had shown that changes made in the flow and wavelength did not

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produce significant changes in analytical results which were presented in the above table. As the changes are not significant, we can say that the method is robust.

System suitability

The system suitability parameters such as US tailing factor, US theoretical Plates and resolution was achieved by injecting the prepared solution five times individually into the chromatographic system separately. The data was shown in Table.

Table No.8: Standard Results of Timolol Maleate and Dorzolamide Hydrochloride

S. No	Injections	RT	Area	USP plate Count	USP tailing
		TM	DH	TM	DH
1.	Injection 1	8.373	6.558	1938696	1448954
2.	Injection 2	8.372	6.560	1958364	1463233
3.	Injection 3	8.372	6.560	1909171	1424815
4.	Injection 4	8.372	6.560	1942383	1451382
5.	Injection 5	8.372	6.560	1894348	1409707

Table No. 9 : System suitability data of Timolol Maleate and Dorzolamide Hydrochloride

Parameter	Timolol Maleate	Dorzolamide Hydrochloride	Acceptance criteria
Retention time (min)	8.372	6.560	±10
Theoretical plates	4728	9459	>3000
Tailing factor	1.17	1.18	<2.00
% RSD	1.35	1.51	<2.00

Results of system suitability study are summarized in the above Table. Five consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

Specificity:

The chromatograms of standard and sample are identical with nearly same retention time. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific.

Table No.10: Specificity data for Timolol Maleate and Dorzolamide Hydrochloride

Sr. No	Sample Name	RT (min) Timolol Maleate	Rt (min) Dorzolamide Hydrochloride
1	Standard	8.373	6.558
2	Sample	8.372	6.572
3	Blank	-	-
4	Placebo	-	-

Chromatograms explain that retention time for standard, sample and commercial product of Timolol Maleate and Dorzolamide Hydrochloride were same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

TableNo.11: Summary of Simultaneous method development of Timolol Maleate and Dorzolamide Hydrochloride

S.No.	Parameter	Timolol Maleate	Dorzolamide Hydrochloride	Acceptance Criteria
1	System suitability Theoretical plates Asymmetry Retention time (min) %RSD	4728 1.17 8.372 1.35	9459 1.18 6.560 1.51	Not less than 3000 Not more than 1.5 Not more than 2.0
2	Specificity	Specific	Specific	Specific
3	Method precision(%RSD)	0.54	0.35	Not more than 2.0%
4	Linearity parameter	5-25 µg/ml	10-50 µg/ml	

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	Correlation co-efficient (r^2)	0.9991	0.9992	Not less than 0.999
5	Accuracy (Mean % recovery) 50% 100% 150%	99.4 99.7 99.8	99.2 99.7 100.0	97 - 103%
6	Robustness	All the system suitability parameters are within the limits.	All the system suitability parameters are within the limits.	

CONCLUSION:

The present study successfully developed and validated a simple, precise, and robust analytical method for the simultaneous estimation of Timolol Maleate and Dorzolamide Hydrochloride using UV spectrophotometry and HPLC. The developed method is accurate, reproducible, and reliable, making it suitable for routine quality control, stability testing, and simultaneous quantification of Timolol Maleate and Dorzolamide Hydrochloride in pharmaceutical formulations. This validated approach provides a valuable analytical tool for ensuring the safety and efficacy of ophthalmic preparations containing these drugs.

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