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International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.5, pp 1429-1435, Sept-Oct 2014

Development and validation of stability indicating reverse phase high performance liquid chromatography method for Timolol Maleate

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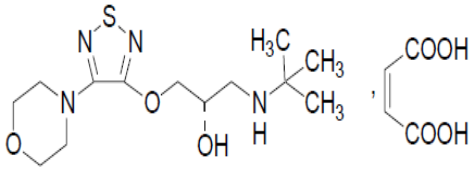
Abstract: A simple, economic , accurate and precise stability indicating method for Timolol Maleate was developed as per ICH guidelines Q1A(R2) and subsequently validated as per ICH guidelines Q2R(1). Chromatographic evaluation was carried out on C_{18} column of Kromasil (250 mm × 4.6 mm, 5µm particle size) with a mobile phase consist of phosphate buffer : methanol (60:40 v/v). The pH of buffer was maintained to 3.5 by using O-phosphoric acid. Flow rate was maintained to 1ml/min. Timolol Maleate was found to be stable in all conditions except in alkaline condition. In alkaline condition two degradation products were observed. Method was validated for different parameters like Linearity (calibration curve), accuracy, precision, robustness, recovery study. LOD and LOQ was found to be 0.724 and 2.19µg/ml respectively. The optimized and validated method can be used for estimation of Timolol Maleate in bulk and also in finished product
KeyWords: Timolol Maleate, Stability studies, reverse phase- high performance liquid chromatography

1. Introduction

Timolol maleate (TM), is a non-selective beta-adrenergic receptor blocking agent that lowers the ocular pressure in open angle glaucoma and ocular hypertension[1]. It is chemically described as (S)-1-(tert-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-propan-2-ol Maleate[2] which is official in U.S.P, I.P and E.P[3]. It was introduced for clinical use in the management of glaucoma in 1978 since then none of the newer beta blocker have been found more effective than Timolol Maleate[4,5]. In salt form, it has a molecular formula $C_{13}H_{24}N_4O_3S$, $C_4H_4O_4$ with molecular weight of 432.50 and pKa 9.21. It is white, odorless, crystalline powder with hydrophilic in nature having melting point 202 ± 0.5°C[4,6]. Structure of Timolol Maleate is shown in Figure 1.

Figure 1. Chemical Structure of Timolol Maleate



Stability testing forms an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under a variety of environmental conditions like temperature, humidity, light etc. Stability studies helps to reveal storage conditions and shelf life of drug. It must be performed as per guidelines of International Conference on Harmonization (ICH)[7].

Various HPLC methods have been reported for simultaneous estimation of Timolol Maleate in combination of other antiglaucomal drugs [2, 3, 4, 8]. A literature survey revealed that very few methods were developed and none of the reported procedures enables analysis of the Timolol Maleate in pharmaceutical dosage forms in the presence of their degradation products. Present work describes the development and validation of a rapid, economical, precise and accurate stability-indicating isocratic RP-HPLC method for analysis of Timolol Maleate in the presence of its degradation products in accordance with ICH guidelines.

2. Materials and Method

2.1 Chemicals and solutions

Timolol Maleate was obtained as gift sample from FDC Ltd, Mumbai, India. Methanol (HPLC grade), sodium hydroxide (NaOH), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were purchaced from Qualigens Fine Chemicals, Mumbai. The 0.45 µm membrane filter was obtained from Pall Lab Sci, India. Freshly prepared Double distilled water was used throughout experiment. All other chemicals used were of analytical HPLC grade.

2.2 Instrumentation

Chromatographic separation was performed by using Jassco HPLC model containing PU-2080 isocratic pump, equipped with UV detector and rheodyne injector with 20 µl loop. Chromatographic analysis was performed by using Borwin chromatography software. Contech CB-50 analytical balance, ultra sonic cleaner (Spectra lab, UCB-40) and Jasco, V-630 UV spectrophotometer were used during the study.

2.3 Preparation of solutions

2.3.1 Preparation of stock solution of drug

Timolol Maleate (100 mg) was weighed and transferred into 100 ml volumetric flask. It was allowed to dissolve in 50 ml of HPLC grade Methanol and volume was made upto 100 ml to get the solution concentration 1000µg/ml.

2.3.2 Phosphate buffer (0.01M)

Accurately weighed potassium dihydrogen phosphate (0.680 gm) was transferred to a beaker and dissolved in 500 ml double distilled water. The pH of buffer was adjusted to 3.5 by o-phosphoric acid.

2.3.3 Diluents

Mixture of Methanol and potassium dihydrogen phosphate buffer (pH adjusted to 3.5) in the ratio 40:60 v/v was used as diluents for dilution of samples.

2.4 Estimation of wavelength of maximum absorbance

To determine λ_{max} of Timolol Maleate, stock solution was suitably diluted to get a final solution of concentration 10µg/ml. UV spectrum was recorded over the wavelength range 200-400 nm.

2.5 Chromatographic conditions

The process was carried out on C_{18} column of Kromasil (250 mm × 4.6 mm, 5µm particle size). Mobile phase consisted of phosphate buffer (pH 3.5): methanol (60:40 v/v). The mobile phase was filtered through a 0.45 µm membrane filter and degassed prior to use. The flow rate was set at 1.0 ml/min. Elution was measured at 295 nm.

2.6 Stability study

Timolol Maleate was subjected for forced degradation study to check stability under various stressed conditions of acid, base, oxidation and thermal as per ICH guidelines Q1A (R2)[9].

2.6.1 Acid degradation study

To study the effect of acid, 1ml stock solution (1000 µg/ml) of TM was pipette out in 10 ml volumetric flask containing 1ml of 1M hydrochloric acid (HCl) and heated at 80°C for various time intervals of 2hr-12hr on water bath. The solution was then left to reach ambient temperature and neutralized to pH 7 by addition of 1M sodium hydroxide (NaOH) then diluted to 10 ml with diluents and injected in RP-HPLC.

2.6.2 Alkali degradation study

Effect of alkali was studied by placing 1ml stock solution (1000 µg/ml) of TM in 10 ml volumetric flask containing 1ml of 1M sodium hydroxide (NaOH) and heated at 80°C for various time intervals of 2hr-12hr on water bath. The solution was then left to reach ambient temperature and neutralized to pH 7 by addition of hydrochloric acid (HCl) then diluted to 10 ml with diluents and injected in RP- HPLC.

2.6.3 Oxidative degradation

Oxidative degradation study was carried out by using hydrogen peroxide. 1ml stock solution (1000 µg/ml) of TM was pipette out in 10 ml volumetric flask and mixed with10%, 15% and 30% H_2O_2 separately. Sample was heated at 80°C for approximately 2hr-12hr on water bath. The solution was then left to reach ambient temperature and diluted to 10 ml with diluents and injected.

2.6.6 Thermal degradation

To check effect of temperature on stability of TM, approximately 10 mg Timolol Maleate was stored at 80°C in hot air oven for required time interval. Simultaneously, another drug containing flask was kept at room temperature as control. After required exposure, drug was dissolved in diluents and volume was adjusted upto mark by diluents and injected into RP-HPLC.

2.7 Method validation

The method was validated according to ICH guideline Q2R(1)[10].

2.7.1 Calibration curve

ICH recommends that for establishment of calibration curve, a minimum of five concentrations should be used. To set calibration curve, stock solution of drug (1000µg/ml) was further diluted with the help of diluents in concentration range of 10-50 µg/ml. The samples were injected in triplicate into the RP-HPLC. A standard plot of peak area v/s concentration of drug in µg/ml was plotted. Correlation coefficient and regression equation were obtained from the calibration curve.

2.7.2 Accuracy

With reference to ICH guideline accuracy should be assessed by using a minimum of nine determinations over a minimum of three concentration level. Accuracy of method was established by diluting stock solution of drug with diluents to get 25, 35, 45 µg/ml drug concentrations. The samples were injected in triplicate into the RP-HPLC. Accuracy of given analytical method were calculated.

2.7.3 Precision

As per ICH guidelines precision should be assessed using a minimum of nine determinations covering the specific range for procedure or using a minimum of six determinations at 100% of test concentration. Repeatability of the method was established by making triplicate injections of three samples generated at three different concentration levels, viz. 30, 40, 50 µg/ml on the same day. The values of percent relative standard deviations (%RSD) were calculated.

2.7.4 Robustness

To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized method parameters were done. The effect of change in flow rate (±0.2 ml/min), mobile phase composition (± 2ml) and wavelength (±2nm) on the area of chromatograms were studied.

2.7.5 Recovery

Recovery of the developed method was evaluated by spiking a formulation solution with three known concentrations of the drug. Formulation containing 1500µg/ml drug concentration which was diluted with diluent to prepare 30µg/ml concentration and injected in triplicate into RP-HPLC. Further three known concentration of drug viz. 24, 30, 36µg/ml was spiked into 30µg/ml of formulation and volume was adjusted by diluent. Samples were injected into HPLC and recovery of added drug was determined.

2.7.6 Limit of detection (LOD)

Limit of detection is the minimum quantity of the drug which can be detected by the method. Limit of detection is calculated as

$LOD=3.3\ (\sigma/S)$

Where σ is the standard deviation of the constant and S is the mean of slope of the calibration curve equation.

2.7.7 Limit of quantification (LOQ)

Limit of quantification is the minimum quantity of the drug that can be quantified by the method. Limit of quantification is calculated as

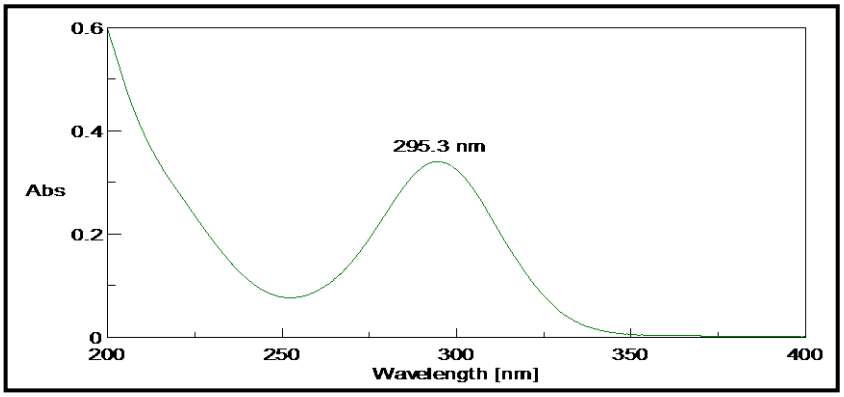
LOQ = 10 (σ/S)

3.0 Results and discussion

3.1 Estimation of wavelength of maximum absorbance

The λ_{max} of drug was found to be 295.3 nm . A UV spectrum of drug is shown in Figure 2.

Figure 2. λ_{max} of Timolol Maleate



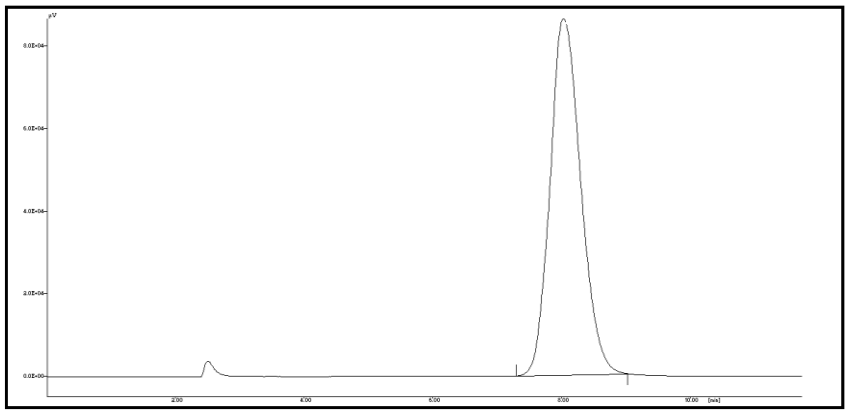
3.2 Method optimization

3.2.1 Selection and Optimization of Mobile Phase for RP-HPLC method development

The aim was to develop simple, economical and precise RP-HPLC method. Method development was carried out according to literature survey. Different trials were carried out by using different combinations of potassium dihydrogen phosphate (pH = 3.5) and methanol. For each trial 100µg/ml drug concentration was used.

The first trial was conducted by using mobile phase in ratio 40:60 (buffer : methanol) which shows drug retention time 4.967 min. Although sharp peak were observed with this composition but drug retention time was not suitable for stability study. Next trial was made by changing mobile phase composition to 60:40 which shows sharp peak at 8.025 min with stable base line. A typical chromatogram is shown in Figure 3.

Figure 3. Chromatogram of Timolol Maleate



3.2.2 System suitability

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N), tailing factor (T_f), and peak asymmetry (A_s) were evaluated for five replicate injections of Timolol Maleate at a concentration of 100 µg/ml. The results given in Table No. 1 were within acceptable limits.

Table No. 1 System suitability studies results

Parameter	Value	Limit
Retention time (Rt)	8.025±0.5	-
Theoretical plates (N)	5669	N > 2000
Tailing factor (T_f)	1.00	$T \leq 2$
Asymmetry (A_s)	1.19	$A_f \leq 2$

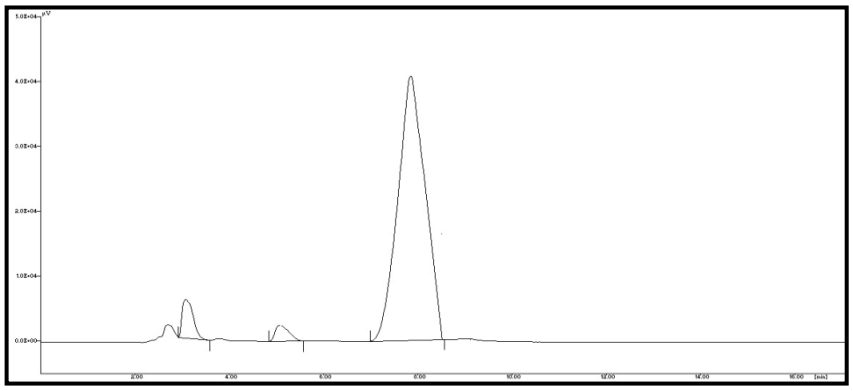
3.3 Forced degradation studies

Timolol Maleate was subjected to forced degradation study as per ICH guidelines. Drug was found to be stable in all condition except in basic environment. Result obtained during alkali degradation studies are shown in Table No. 2 and Figure 4.

Table No. 2 Result from analysis of samples by alkaline degradation study

Parameter/Stress condition/duration	Retention time		Total % degradation
	A	B	
Alkali/ 1N NaOH/ 4hr	3.075	5.067	29.68

Figure 4. Typical chromatogram obtained after degradation of Timolol Maleate under alkaline condition.



3.4 Method Validation

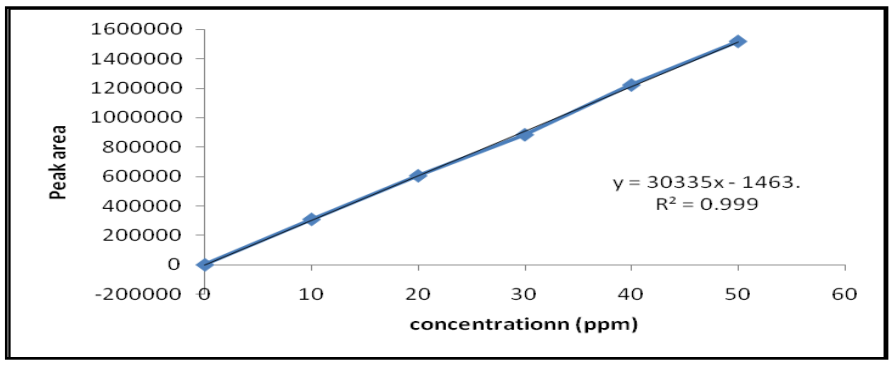
3.4.1 Linearity

The linearity was established in the concentration range of 10-50 µg/ml. Sample of each concentration was injected in triplicate. Result is shown Table No. 3. A standard plot of peak area v/s concentration of drug in µg/ml is shown in Figure 5. The linear regression equation was found to be y = 30379x – 3072 with correlation coefficient (R^2) 0.999.

Table No. 3 Linearity study of Timolol Maleate

Sr. No.	Concentration (µg/ml)	Mean peak area	%RSD
1	10	309149	1.079
2	20	605636	1.373
3	30	884307	0.261
4	40	1223016	1.117
5	50	1519427	0.169

Figure 5. Calibration curve of Timolol Maleate



3.4.2 Accuracy

Accuracy of analytical method was determined by three concentration of drug viz. 25, 35, 45 µg/ml and injected in triplicate. Results are shown in Table No. 4. Accuracy was found to be within the acceptable limit of 98-102%.

Table No. 4 Accuracy studies

Sr. No.	Concentration (µg/ml)	Mean peak area	%RSD	Concentration found	Accuracy
1	25	697231	1.28	25.203	100.81
2	35	973693	1.07	34.593	98.83
3	45	1286034	1.71	45.203	100.45

3.4.3 Precision

Precision of analytical method was determined by injecting three concentration of drug viz. 30, 40, 50µg/ml in triplicate. Data obtained during precision study is shown in Table No. 5. Percent RSD was found to be less than 2%.

Table No.5 Precision Study

Sr. No.	Concentration (µg/ml)	Mean Peak Area	% R.S.D
1	30	877061	1.337
2	40	1167645	0.886
3	50	1440323	0.452

3.4.4 Robustness

Method was found to be unaffected by small deliberate changes in mobile phase composition, wavelength and flow rate. Results are shown in Table No.6

Table No.6 Robustness study

Parameter		Peak Area	RSD (%)
Wavelength	293 nm	1439250	0.629
	297 nm	1430363	1.951
Flow rate	0.8 ml/min	1832813	1.270
	1.2 ml/min	1182131	0.525
Method	58:42 ml	1199127	1.807
	62:38 ml	1152018	1.447

3.4.5 Recovery study

Recovery of the developed method was evaluated by spiking a formulation solution with three known concentrations of the drug. Results of recovery study are shown in Table No.7. Drug recovery was found to be within acceptable range (98-102%) as per ICH guidelines.

Table No. 7 Recovery study

Mean peak area		Concentration recovered	% Recovery
Formulation + Spiked drug	Formulation		
1602908	871677	24.17	100.71
1791474	871677	30.37	101.26
1941798	871677	35.32	98.12

3.4.6 Limit of detection (LOD)

Limit of detection of Timolol Maleate was found to be 0.724µg/ml.

3.4.7 Limit of quantification (LOQ)

Limit of quantification of Timolol Maleate was found to be 2.19µg/ml.

4.0 Conclusion

A stability-indicating RP-HPLC method of Timolol Maleate was developed and validated as per ICH guidelines. The degradation behaviour of Timolol Maleate was studied under various stress conditions of acid, alkali, oxidation and temperature. Drug was found to be unstable in basic condition. Two degradation products were observed in alkaline stress study. The developed method is rapid, economic, accurate and precise for quantitative analysis of Timolol Maleate in bulk and also in finished product.

Acknowledgment

Authors are thankful to Dr. C. R. Kokare, Principal, SIOP (Pune) for providing all the facilities. The authors duly acknowledge FDC Ltd., Mumbai, India, for providing Timolol Maleate as a gift sample.

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