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Development and Validation of Stability Indicating Analytical Method for Simultaneous Estimation of Timolol Maleate and Pilocarpine Nitrate in Combined Dosage Form

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ABSTRACT

A simple and effective stability indicating RP-HPLC method for simultaneous estimation of Timolol Maleate and Pilocarpine nitrate in combined dosage form has been developed and validated**.** The chromatographic separation was carried out on Shim-pack solar C18 (250 × 4.6mm, 5µm), mobile phase Acetonitrile:water (50:50 v/v), detection wavelength at 230 nm, at flow rate of 0.9 ml/min at retention time 2.454 min for Timolol maleate, 5.541 min for Pilocarpine nitrate. Linearity was obtained in the range of 30µg/ml to 70µg/ml for Timolol Maleate and 120µg/ml to 280µg/ml for Pilocarpine nitrate, respectively. The correlation coefficient was found to be 0.9911 for Timolol maleate and 0.9923 for Pilocarpine nitrate. The % Assay for Timolol Maleate is 99.66 % and Pilocarpine nitrate is 99.87 %. Forced Degradation studies were conducted according to the ICH guidelines and the Drug Product was found to be stable in all conditions. Hence, the method could be successfully applied for routine analysis of Timolol Maleate and Pilocarpine nitrate in combined dosage form.

INTRODUCTION

Glaucoma is characterized by an increase in intraocular pressure (IOP) which, if persistent, can lead to damage to optic disc resulting in blindness. Glaucoma is caused by the impaired drainage of aqueous humour which is produced by the ciliary epithelium in the posterior chamber of the eye.[1-2]Chemically Timolol maleate(S)-1-(tert-butylamino)-3-(4-morpholino-1,2,5-thiadiazol-3-yloxy)propan-2-ol hydrogen maleate is a Beta-adrenoceptor antagonist (Antiglaucoma).[3] It is usually given in eyedrop form. Timolol maleate is a beta1 and beta 2 (non-selective) adrenergic receptor blocking administrator that doesn't have critical characteristic sympathomimetic, direct myocardial depressant, or nearby sedative (membrane-stabilizing) action. Ophthalmic timolol is indicated for the treatment of increased intraocular pressure in patients with ocular hypertension or open-angle glaucoma. The oral form of this drug is used to treat high blood pressure.[4] Pilocarpine nitrate is (3*S*,4*R*)-3-ethyl-4-[(1-methyl-1H-imidazol-5-yl)methyl]dihydrofuran-2(3H)-one nitrate is a Cholinergic (ophthalmic) in glaucoma.[5] Pilocarpine is a cholinergic parasympathomimetic agent. It increases secretion by the exocrine glands and produces contraction of the iris sphincter muscle and ciliary muscle (when given topically to the eyes) by mainly stimulating muscarinic receptors. Pilocarpine is utilized as a miotic and in the treatment of glaucoma.[6]

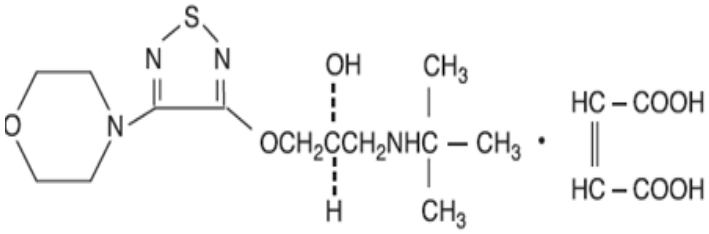

Figure No. 1: Structure of Timolol maleate

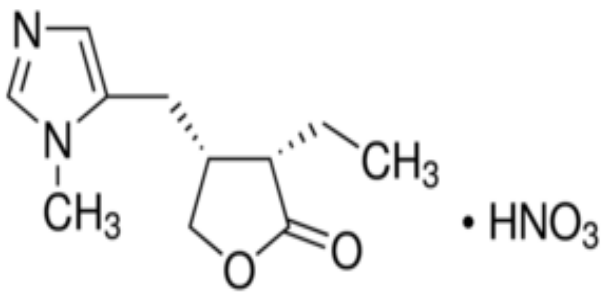

Figure No. 2: Structure of Pilocarpine nitrate

The combination of Timolol maleate and Pilocarpine nitrate is very useful in the treatment of glaucoma. On literature survey, it was found that spectroscopic method and HPLC method has been reported for the simultaneous estimation of Timolol maleate and Pilocarpine nitrate in combined dosage forms. In the view of the need for a suitable method for routine analysis in combined formulations, attempts are being made to develop simple, precise and accurate analytical methods for simultaneous estimation of titled ingredients and extend it for their determination in formulation.[7-9] **Forced degradation:** The regulatory agencies have defined the limits of degradation products in their guidelines. It is mentioned that 5–20% degradation is accepted validation of chromatographic assays. In case of small molecules, stability limit should be more than 90% and hence about 10% degradation is sufficient. In general, for monitoring drug product stability, spiked samples of mixture of known degradation products and drug substances are used, which ease out the process of determining the products that are observed during the degradation.[10-11]

MATERIALS AND METHODS

Reagents and materials

Analytically pure sample of Timolol maleate and Pilocarpine nitrate were obtained as gift samples from FDC LTD (Mumbai) and eye drop purchase from local market. Distilled water (HPLC grade-RANKEM), Acetonitrile (HPLC grade-RANKEM), Methanol (HPLC grade-Fisher RANKEM), Sodium hydroxide (NaOH), Hydrochloric acid (HCl), Hydrogen peroxide (H_2O_2).

Instrument

HPLC analysis was performed on Shimadzu LC-2010C HT, Autosampler, UV detector and enable column Shim-pack solar C18 (250 × 4.6mm, 5µm), Autoinjector with 10µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "LC solution" software. In addition, an electronic weighing balance **(**Reptech-0.1mg) digital pH meter **(**Systronics pH system 361), a sonicator (Athena technology) and UV spectrophotometer (Shimadzu 1800 series, Software UV probe version 2.42) were used in this study. Filter paper is membrane filter 0.45 micron.

Selection of Wavelength

In present study, individual drug solutions of 5µg/ml of Timolol maleate and 20 µg/ml of Pilocarpine nitrate were prepared in Methanol. These drugs solutions were scanned in the UV region of 200-400 nm and spectrum was recorded. Wavelength was selected from overlay spectra of Timolol maleate and Pilocarpine nitrate.

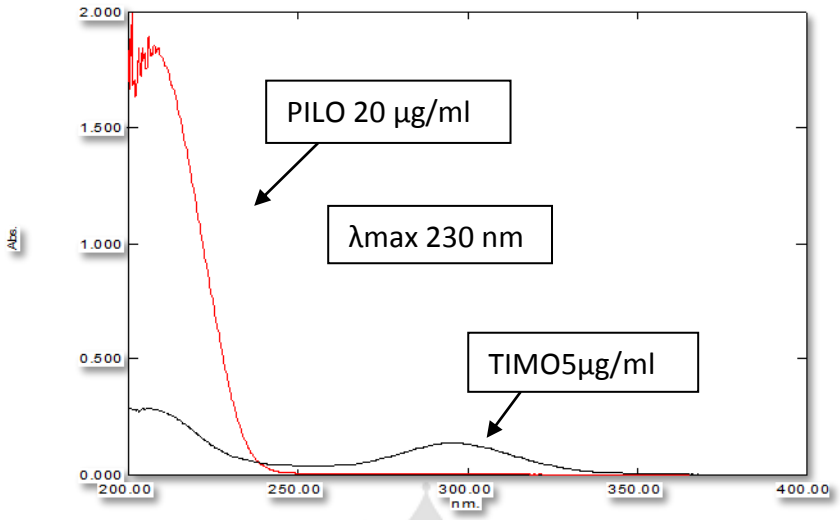

Figure No. 3: Overlay spectra of TIMO and PILO

CHROMATOGRAPHIC CONDITIONS

The separation of the drugs was achieved on a C18 column reverse phase (250 × 4.6mm, 5µm). The mobile phase consists of a mixture of Acetonitrile: Water as the mobile phase in the ratio 50:50 v/v at flow rate 0.9 mL/min and volume injected was 10µL for every injection the detection wavelength was set at 230 nm.

PREPARATION OF STANDARD SOLUTION

1. Preparation of TIMO standard stock solution (1000 µg/ml)

10 mg of TIMO was weighed and transferred to 10 ml volumetric flask. It was dissolved in methanol and volume was made up to the mark with methanol to give a solution containing 1000 µg/ml. Aliquot of 5 ml from above standard stock solution was pipette out into 25 ml of volumetric flask and volume was made up to the mark with methanol to give 200 µg/ml.

2. Preparation of PILO standard stock solution (1000 µg/ml)

25 mg of PILO was weighed and transferred to 25 ml volumetric flask. It was dissolved in methanol and volume was made up to the mark with methanol to give a solution containing 1000 µg/ml. Aliquot of 12.5 ml from above standard stock solution was pipette out into 25 ml of volumetric flask and volume was made up to the mark with methanol to give 500 µg/ml.

3. Sample preparation

To take a 1 ml of liquid containing 5 mg TIMO and 20 mg of PILO was transferred to 10 ml volumetric flask then the volume was made up to the sufficient with methanol to get 100 µg/ml concentration. Shaking was carried out for 5 min. at that point, arrangement was filtered through whatman filter paper. Form the 100 µg/ml of sample solution take 1 ml of solution and further diluted up to the mark in 10ml volumetric flask. So the final solution was made which contain 20 µg/ml Pilocarpine nitrate and 5 µg/ml Timolol maleate both. The solution was scanned from 400-200 nm. The concentration of both TIMO and PILO were determined by measuring absorbance sample solution at 230 nm.

FORCED DEGRADATION CONDITION[12]

1. ACID DEGRADATION

To study the effect of acid, 2ml stock solution (100µg/ml) of TIMO and PILO was pipette out in 10 ml volumetric flask containing 2ml of 0.1N HCl and heated at 80°C for various time intervals of 1hr-24hr on water bath. The solution was then left to reach ambient temperature and neutralized to 0.1 N NaOH then diluted to 10 ml with diluents to get 10µg/ml TIMO and 40 µg/ml of PILO and injected in RP-HPLC. The obtained chromatogram shown in Fig.7.

2. BASE DEGRADATION

To study the effect of base, 2ml stock solution (100 µg/ml) of TIMO and PILO was pipette out in 10 ml volumetric flask containing 2ml of 0.1 N NaOH and heated at 80°C for various time intervals of 1hr-24hr on water bath. The solution was then left to reach ambient temperature and neutralized to 0.1N HCl then diluted to 10 ml with diluents to get 10µg/ml TIMO and 40 µg/ml of PILO and injected in RP-HPLC. The obtained chromatogram shown in Fig. 8.

3. NEUTRAL DEGRADATION

To study the effect of neutral, 2ml stock solution (100 µg/ml) of TIMO and PILO was pipette out in 10 ml volumetric flask containing 2ml of HPLC grade water and heated at 80°C for various time intervals of 1hr-24hr on water bath. The above solution, 2 ml of working solution was pipette out into 10 ml volumetric flask, and finally made up to volume with mobile phase to get 10 µg/ml TIMO and 40 µg/ml of PILO. The obtained chromatogram shown in Fig. 9.

4. OXIDATIVE CONDITION

Oxidative degradation study was carried out by using hydrogen peroxide, 2ml stock solution (100µg/ml) of TIMO and PILO was pipette out in 10 ml volumetric flask containing 2ml of 3% H_2O_2 at room temperature for a defined period of time to get 10µg/ml TIMO and 40 µg/ml of PILO and injected in RP-HPLC. The obtained chromatogram shown in Fig. 10.

5. THERMAL DEGRADATION

25 mg of TIMO and 100 mg of PILO drug mixture was exposed to dry heat at 80°C for 10 weeks. From the above sample, at specified time interval 1.5 mg of drug mixture was accurately weighed in 10 ml volumetric flask and made up to mark with methanol to get 30µg/ml of TIMO and 120 µg/ml of PILO. From the above solution 2 ml of solution was pipette out in 10 ml volumetric flask and made up to mark with mobile phase ration to get 6 µg/ml of TIMO and 24 µg/ml of PILO. The obtained chromatogram shown in Fig. 11.

6. PHOTO DEGRADATION

25 mg of TIMO and 100 mg of PILO drug mixture was spread in 1 mm thickness uniform layer on petridish and exposed in sunlight for some days. From the above sample, at specified time interval 1.5 mg of drug mixture was accurately weighed in 10 ml volumetric flask and made up to mark with methanol to get 30µg/ml of TIMO and 120 µg/ml of PILO. From the above solution 2 ml of solution was pipette out in 10 ml volumetric flask and made up to mark with mobile phase ration to get 6 µg/ml of TIMO and 24 µg/ml of PILO. The obtained chromatogram shown in Fig. 12.

METHOD VALIDATION[13]

1) System Suitability studies

Evaluation of system suitability was done by analyzing six replicate of TIMO and PILO in a mixture at concentration of 50 µg/ml of TIMO and 200 µg/ml of PILO. The column efficiency, peak asymmetry and resolution were calculated for each replicate. (Table 1)

2) Specificity

Specificity involves quantitative detection of analyte in the presence of those components that may be expected to be part of sample matrix. Specificity of developed method was established by spiking of TIMO and PILO in hypothetical placebo (*i.e.* may be relied upon to be present) and expressing that analytes peak were not interfered from excipients.

3) Linearity and Range (n=5)

The linearity response was determined by analyzing 5 independent levels of concentration in the range of 30-70 µg/ml and 120-280 µg/ml for TIMO and PILO respectively. Calibration curves were constructed by plotting absorbance vs concentration and regression analysis was performed. The equation of calibration curves was Y= 14,275.5700x + 88,557.0600 for TIMO and Y=5,054.8300x - 20,708.1600 for PILO respectively (Fig.4, 5) (Table 2, 3).

4) Precision

a. Repeatability (n=6)

Aliquots of 2.5 ml of working stock solution of TIMO (200µg/ml) were transferred to a series of 10 ml volumetric flask. Aliquots of 4.0 ml of working stock solution of PILO (500 µg/ml) were respectively transferred to the same above series of 10 ml volumetric flask. The volume was adjusted up to mark with mobile phase ratio to get 50µg/ml solution of TIMO and 200 µg/ml solution of PILO and % RSD was calculated. (Table 4)

b. Intraday precision (n=3)

Aliquots of 2, 2.5 and 3 ml of working stock solution of TIMO (200µg/ml) were transferred to a series of 10 ml volumetric flask. Aliquots of 3.2, 4.0 and 4.8 ml of working stock solution of PILO (500 µg/ml) were respectively transferred to the same above series of 10 ml

volumetric flask. The volume was adjusted up to mark with mobile phase ratio to get 40, 50 and 60 µg/ml solution of TIMO and 160, 200 and 240µg/ml solution of PILO. Solutions were analyzed thrice (n=3) on the same day within short interval of time and % RSD was calculated. (Table 5)

c. Interday precision (n=3)

Aliquots of 2, 2.5 and 3 ml of working stock solution of TIMO (200µg/ml) were transferred to a series of 10 ml volumetric flask. Aliquots of 3.2, 4.0 and 4.8 ml of working stock solution of PILO (500 µg/ml) were respectively transferred to the same above series of 10 ml volumetric flask. The volume was adjusted up to mark with mobile phase ratio to get 40, 50 and 60 µg/ml solution of TIMO and 160, 200 and 240µg/ml solution of PILO. Solutions were analyzed thrice (n=3) on the same day within short interval of time and % RSD was calculated. (Table 6)

5) LOD and LOQ (n=5)

The LOD (Limit of Detection) was assessed from the set of 5 calibration curves that were used to determine linearity of the method. The LOD and LOQ were calculated as LOD = 3.3 × S.D./Slope and LOQ = 10 × S.D./Slope Where, S.D. = Standard deviation of the Y – intercepts of 5 calibration curves Slope = Mean slope of 5 calibration curves. The LOD of TIMO and PILO were found to be 1.5130µg/ml and 7.2869µg/ml respectively. The LOQ of TIMO and PILO were found to be 4.5849µg/ml and 22.0817µg/ml respectively.

6) Accuracy

The accuracy of the method is the nearness of the obtained to the true value. Accuracy of the method was determined by recovery experiments. Accuracy was determined at three different level 80%, 100% and 120% of the target concentration in triplicate. At each level, three determinations were performed and results obtained. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay. The amounts recovered, values of percent mean recovery were calculated as shown in (Table 7).

7) Robustness

Robustness of the method was determined by subjecting the method to slight change in the method condition like, Mobile Phase Ratio and Flow rate. Three replicates were prepared for

the same of concentration 50µg/ml for TIMO and 200µg/ml for PILO and % R.S.D. was calculated. (Table 8)

RESULTS AND DISCUSSION

1. Method validation

a. System suitability Data

Table No. 1: System suitability data of TIMO and PILO

Drugs	Parameters	Mean ± S.D. (n=5)	% R.S.D
TIMO	Retention time	2.451± 0.007	0.2855
	Theoretical plate	7419.129 ± 12.145	0.1637
	Tailing factor	1.561 ± 0.0105	0.6726
PILO	Retention time	5.552 ± 0.0115	0.2071
	Theoretical plate	6366.396 ± 11.477	0.1802
	Tailing factor	1.845 ± 0.011	0.5962
	Resolution	6.137 ± 0.063799	1.0395

b. Linearity

Table No. 2: Linearity data of TIMO

Sr. No.	Conc. (µg/ml)	Mean Peak Area ± S.D. (n=5)	% R.S.D
1	30	507095.6 ± 747.11	0.1473
2	40	657305.0 ± 1400.9	0.2131
3	50	836789.0 ± 5834.9	0.6973
4	60	921923.2 ± 4958.6	0.5378
5	70	1082994 ± 7094.9	0.6517

Table No. 3: Linearity data of PILO

Sr. No.	Conc. (µg/ml)	Mean Peak Area ± S.D. (n=5)	% R.S.D
1	2	559551.4 ± 4246.83	0.7589
2	4	801838.0 ± 1401.53	0.1747
3	6	1007843.2 ± 5723.06	0.5678
4	8	1241240.4 ± 9249.08	0.7451
5	10	1360816.2 ± 18949.48	1.3925

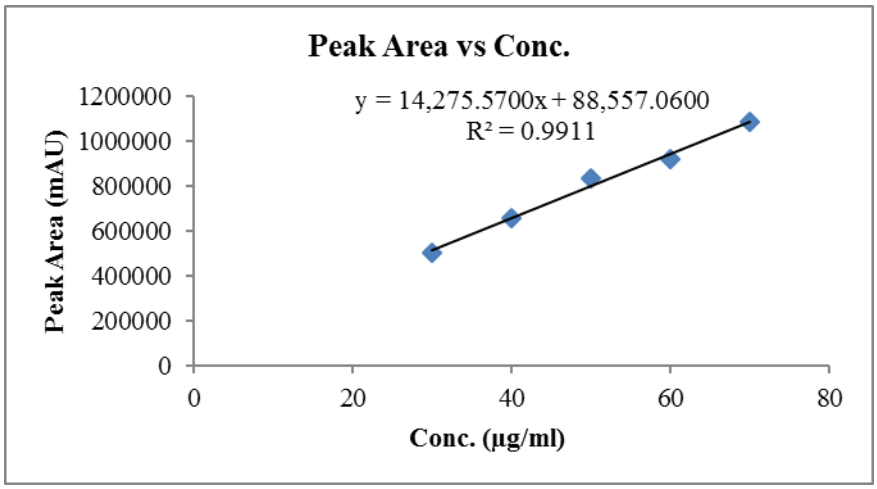

Figure No. 4: Calibration curve of TIMO

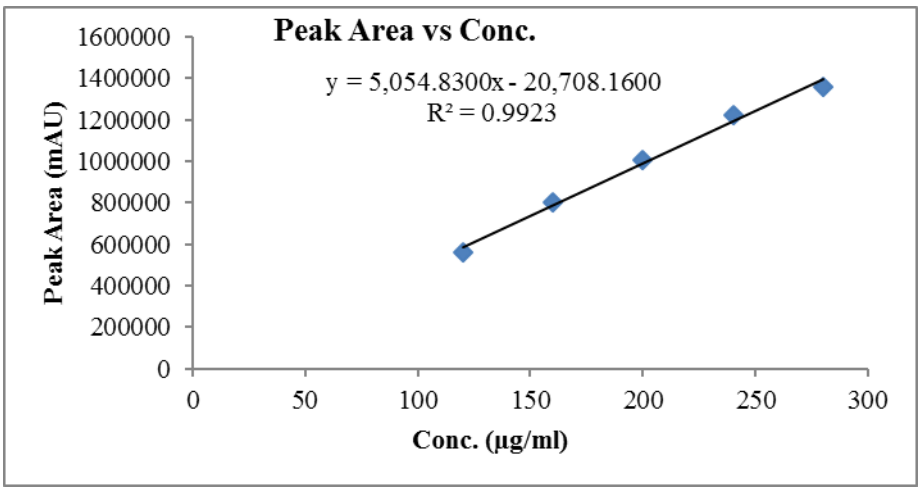

Figure No. 5: Calibration curve of PILO

c. Precision

1. Repeatability

The data of repeatability for TIMO and PILO are depicted in table no. 4.

Table No. 4: Repeatability data of TIMO and PILO

Drugs	Conc. (µg/ml)	Mean Peak Area ± S.D (n=6)	% R.S.D
TIMO	50	846770.3 ± 5957.83	0.7035
PILO	200	1017778.6 ± 5896.58	0.5793

2. Intraday Precision

The data for intraday precision for TIMO and PILO are depicted in the table no.5.

Table No. 5: Intraday data of TIMO and PILO

Drugs	Conc. (µg/ml)	Mean Peak Area ± S.D. (n=3)	% R.S.D
TIMO	40	654860.3 ± 2000.65	0.3055
	50	848641.3 ± 6424.48	0.7570
	60	918548.0 ± 6370.31	0.6935
PILO	160	804572.0 ± 2525.91	0.3139
	200	1018092.6 ± 6871.42	0.6749
	240	1252273.3 ± 9822.49	0.7843

3. Interday Precision

The data for interday precision for TIMO and PILO are depicted in the table no.6.

Table No. 6: Interday data of TIMO and PILO

Drugs	Conc. (µg/ml)	Mean Peak Area ± S.D. (n=3)	%R.S.D
TIMO	40	654627.6 ± 3363.35	0.5137
	50	850041.6 ± 8464.05	0.9957
	60	918663.6 ± 8066.97	0.8781
PILO	160	806971.6 ± 4404.09	0.5457
	200	1020812.0 ± 8746.36	0.8568
	240	1250458.6 ± 12039.10	0.9627

d. Accuracy

Table No. 7: Accuracy data of TIMO and PILO

Drugs	Level	Amt of sample (µg/ml)	Amt of drug spiked (µg/ml)	Total conc. found (µg/ml)	Mean % Recovery
TIMO	0%	30	0	30	98.66 %
	80%	30	24	54	99.81 %
	100%	30	30	60	100.66 %
	120%	30	36	66	101.37 %
PILO	0%	120	0	120	98.66 %
	80%	120	96	216	99.89 %
	100%	120	120	240	100.11 %
	120%	120	144	264	100.17 %

e. Robustness

The Robustness of the method was established by making deliberated minor variations in the following method parameters. Flow rate: ± 0.1 units Mobile Phase: ± 2 units.

Table No. 8: Robustness data of TIMO (50µg/ml)

Parameters	Level	Peak Area ± S.D. (n=3)	% R.S.D	Rt ± S.D. (n=3)	%R.S.D
Mobile Phase (v/v)	48.52	1322076.3 ± 9232.86	0.6983	2.444 ± 0.0144	0.5901
	52:48	1473754.3 ± 12677.04	0.8601	2.543 ± 0.0115	0.4535
Flow Rate (ml/min)	0.8	956794.3 ± 1111.95	0.1162	2.503 ± 0.0222	0.8897
	1	1165154.0 ± 12746.68	1.0939	2.444 ± 0.0226	0.9267

Table No. 9: Robustness data of PILO (200µg/ml)

Parameters	Level	Peak Area ± S.D. (n=3)	% R.S.D	Rt ± S.D. (n=3)	%R.S.D
Mobile Phase (v/v)	48:52	1216196 ± 4592.8	0.3776	5.414 ±0.0464	0.8586
	52:48	1896397.3 ± 16513.8	0.8708	5.561 ± 0.0399	0.7183
Flow Rate (ml/min)	0.8	1013862 ± 2338.8	0.2306	5.230 ± 0.0296	0.5672
	1	1409194 ± 4331.28	0.3073	5.302 ± 0.0098	0.1860

f. Applicability of Proposed method

Table No. 10: Determination of Assay of TIMO and PILO

Eye drop	Amount taken (µg/ml)		Amount obtained (µg/ml)		%TIMO ± S.D (n=3)	%PILO ± S.D (n=3)
	TIMO	PILO	TIMO	PILO		
IOTIM PLUS	50	200	49.83 ± 0.4104	199.742 ± 0.4471	99.66 ± 0.8208	99.87 ± 0.2235

2. Forced Degradation

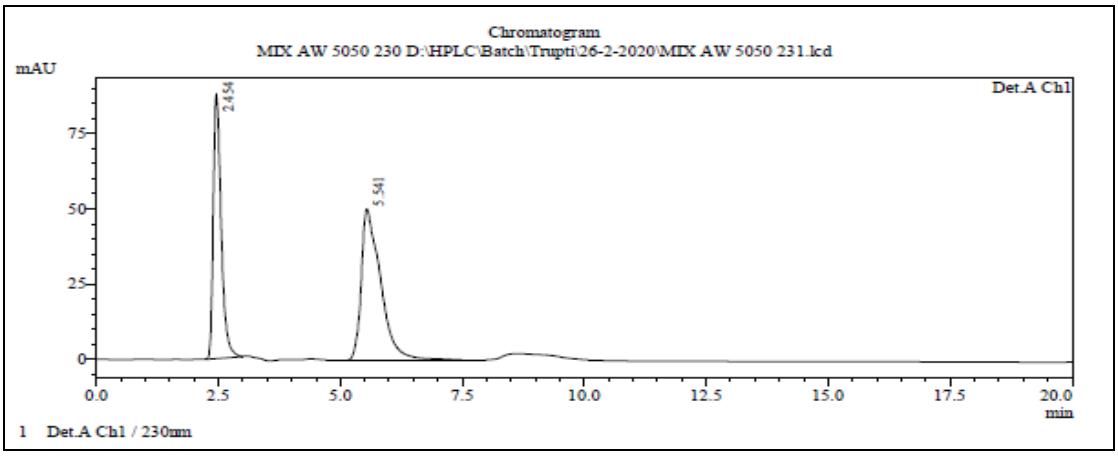

Figure No. 6: Chromatogram of TIMO (50µg/ml) and PILO (200 µg/ml) in ACN: Water (50:50 v/v)

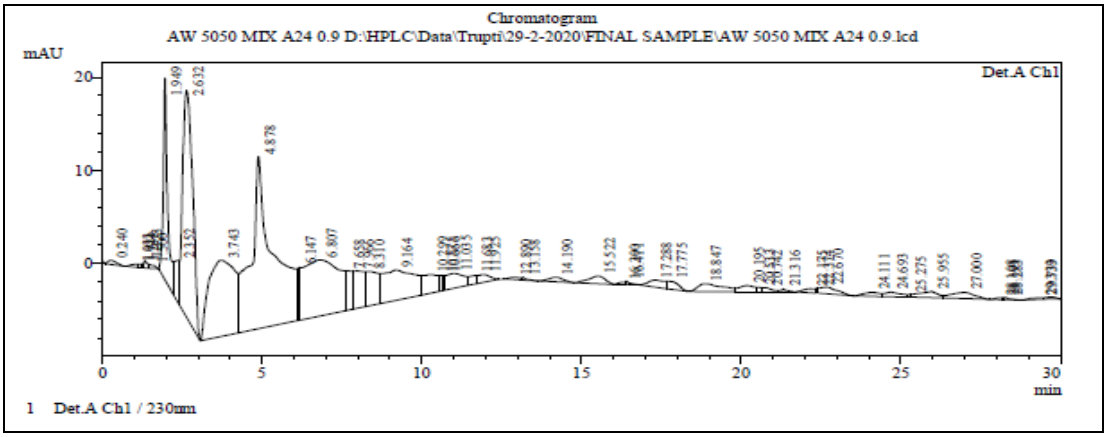

Figure No. 7: Degradation of TIMO and PILO in 0.1N HCl at 24hr

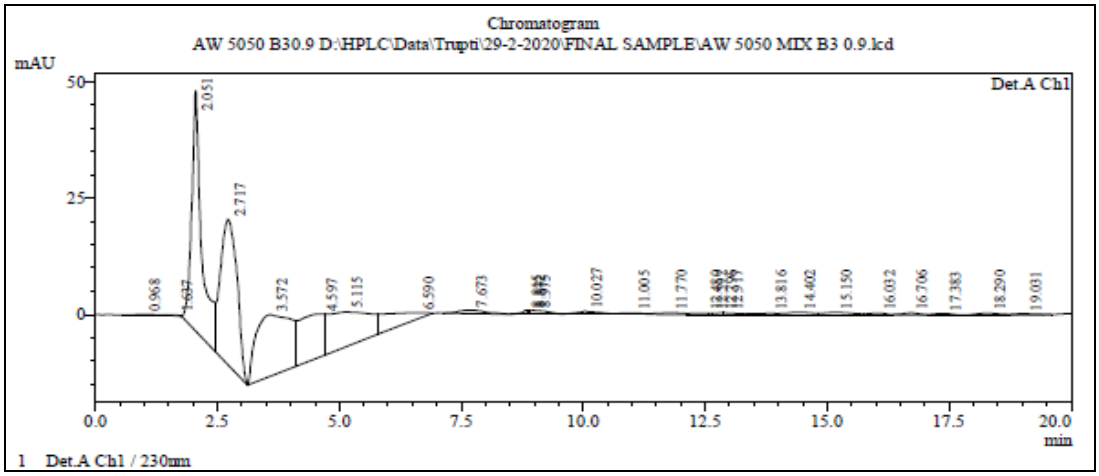

Figure No. 8: Degradation of TIMO and PILO in 0.1N NaOH at 3hr

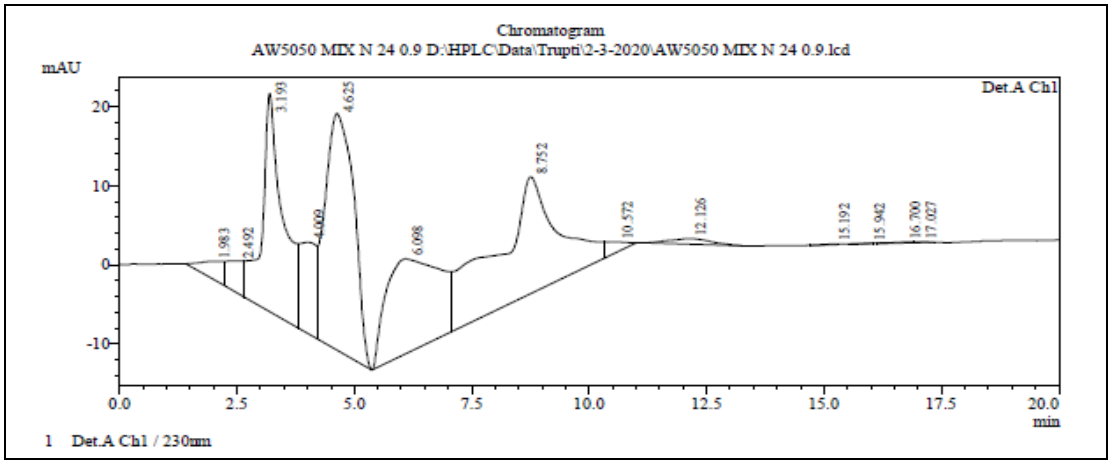

Figure No. 9: Degradation of TIMO and PILO in neutral hydrolysis at 24hr

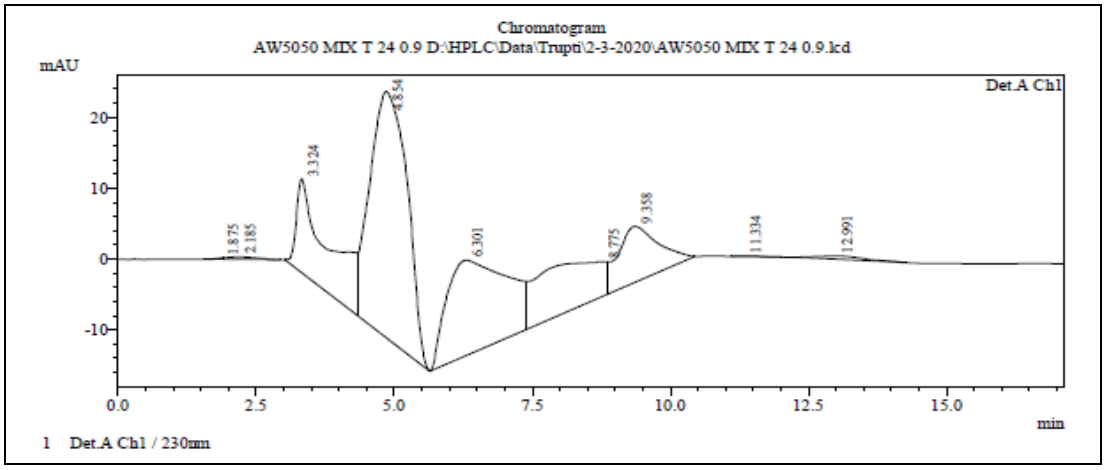

Figure No. 10: Degradation of TIMO and PILO at thermal degradation at 24hr

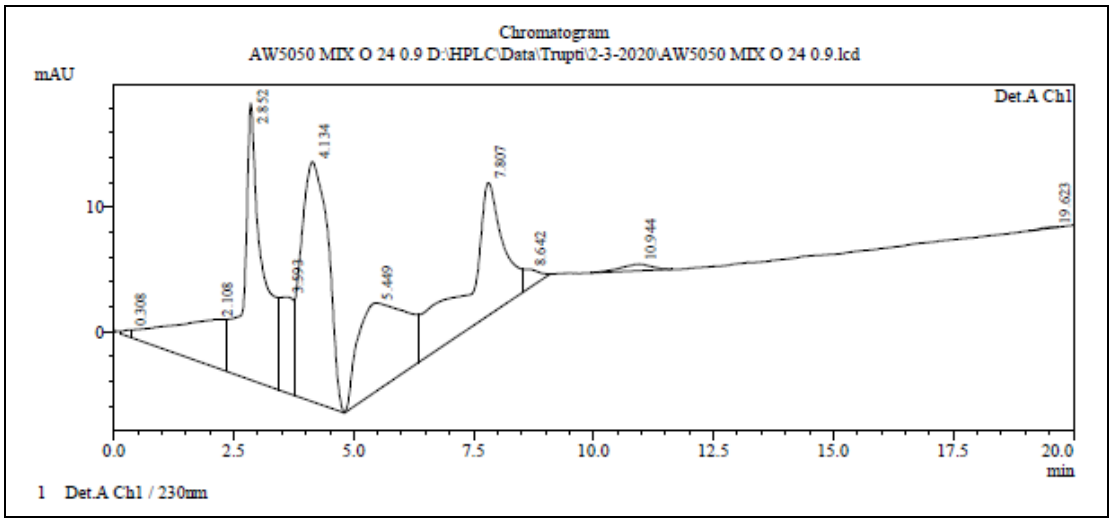

Figure No. 11: Degradation of TIMO and PILO in 3% H_2O_2 in 24hr

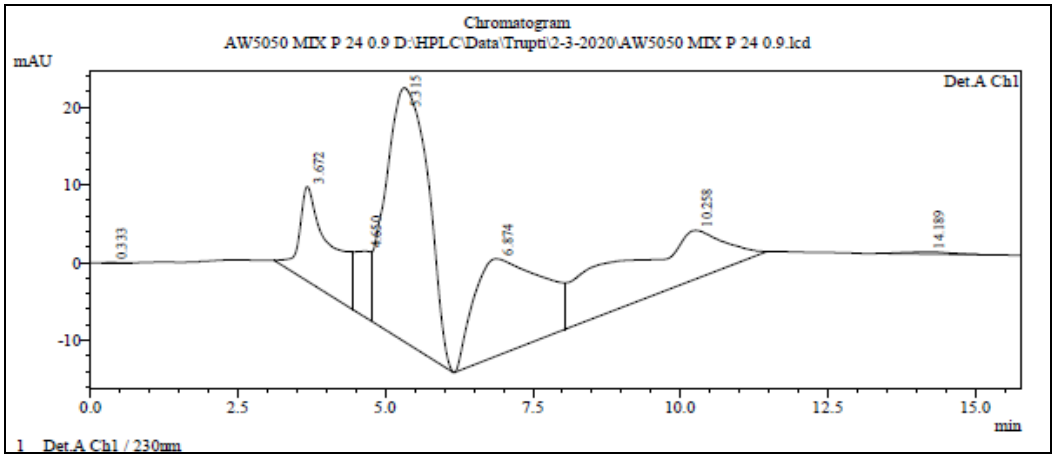

Figure No. 12: Degradation of TIMO and PILO in photodegradation at 24hr

Table No. 11: Forced degradation study

Degradation condition	Time (hr)	Area		% Degradation	
		TIMO	PILO	TIMO	PILO
Control	-	988553	1394287	-	-
0.1 N HCl (Refluxing)	1	435200	888890	55.98	36.25
	24	198977	669584	79.88	51.98
0.1 NaOH (Refluxing)	1	782766	-	20.85	-
	24	639946	-	35.27	-
Neutral HPLC Water (Refluxing)	1	717106	1224936	27.46	12.15
	24	449568	697683	54.53	49.97
Thermal	1	676793	925631	31.54	33.62
	24	549268	477231	51.73	65.78
Oxidative	1	560009	589436	43.36	51.73
	24	286275	324782	71.05	76.71
Photolytic	1	473108	546633	52.15	60.8
	24	340557	370797	65.55	73.41

CONCLUSION

Simultaneous determination of Timolol maleate and Pilocarpine nitrate in their combined dosage form using Stability indicating HPLC has been successfully achieved. The developed method is specific, selective, accurate and precise for reliable quality control evaluation of drugs with good accuracy and precision. Hence, the methods can be used for routine analysis of both the drugs in their combined dosage form.

ACKNOWLEDGEMENT

Authors are very thankful to FDC LTD., Mumbai for providing the gift samples of Timolol maleate and Pilocarpine nitrate, respectively and also wish our sincere thanks to ROFEL Shri G. M. Bilakhia College of Pharmacy, Vapi, for their support and guidance.

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