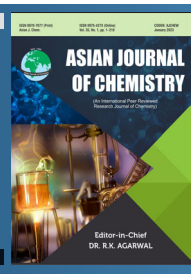
Asian Journal of Chemistry; Vol. 35, No. 1 (2023), 179-186

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2023.26995



A Simple and Validated Stability Indicating HPLC Method for Simultaneous Quantification of Brimonidine, Timolol and Benzalkonium Chloride in Anti-Glaucoma Ophthalmic Formulations

A. KRISHNAMANJARI PAWAR* and CHANDANA MANNEPALLI

A.U. College of Pharmaceutical Sciences, Andhra University, Visakhapatnam-530003, India

*Corresponding author: E-mail: akmpawar@andhrauniversity.edu.in

Received: 16 October 2022; *Accepted*: 13 December 2022; *Published online*: 27 December 2022; AJC-21096

The combined dosage form of brimonidine, timolol and benzalkonium chloride is prescribed for lowering intraocular pressure in patients with ocular hypertension. As no analytical HPLC method reported for the estimation of brimonidine, timolol and benzalkonium chloride together in ophthalmic formulations, the present study was aimed to fulfill the gap identified in literature. The separation of analytes was achieved on Cosmosil C_{18} (250 × 4.6 mm; 5 µ id) as stationary phase, pH 4.3 acetate buffer and methanol in 30:70 (v/v) as mobile phase at 1.0 mL/min and UV detection at 227 nm. In this condition, well resolved, retained peaks were identified at 3.11 min for brimonidine, 4.86 min for timolol and 5.57 min for benzalkonium chloride. The method reports 0.5, 1.25 and 0.0125 µg/mL, respectively for brimonidine, timolol and benzalkonium chloride as LOD, which proves that the method have enough sensitivity levels for the detection analytes in samples. The method passes all the validation parameters as per the guidelines proved that the method was valid. The method shows less % degradation in various stress studies such as acidic, base, peroxide, thermal and UV light conditions and can effectively separate various stress degradation compounds and confirms the stability indicating nature of the method. The method applicability was assessed by analyzing the drug content in Combigan® ophthalmic drops and method reports the assay of 98.92%, 99.36% and 98.46% for brimonidine, timolol and benzalkonium chloride, respectively. Based on the results, it can be concluded that the method can adequately suitable for the separation and quantification of brimonidine, timolol and benzalkonium chloride and hence can be applicable for the routine analysis of brimonidine, timolol and benzalkonium chloride in ophthalmic formulations.

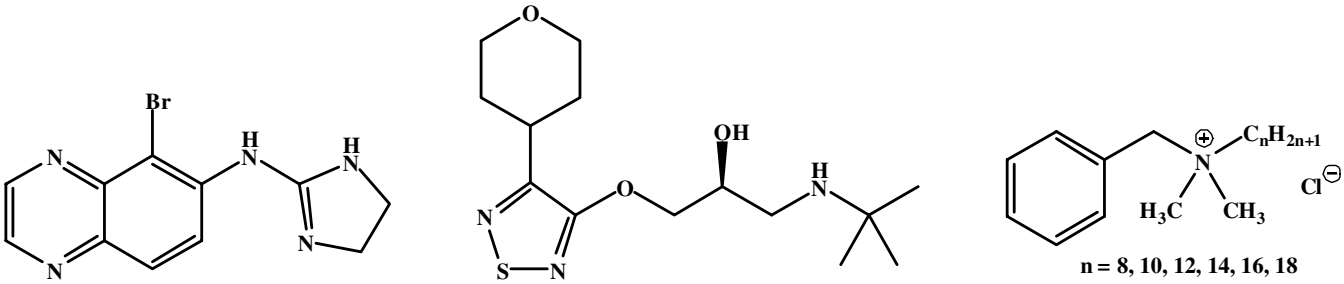
Keywords: Brimonidine, Timolol, Benzalkonium chloride, HPLC analysis, Stress studies, Formulation assay.

INTRODUCTION

Brimonidine (Fig. 1a) is a medical drug that belongs to selective alpha-2 adrenergic receptor agonist and an imidazole derivative prescribed for the treatment of ocular hypertension, open-angle glaucoma and rosacea [1]. Brimonidine also used for lowering the intraocular pressure in patients suffering with ocular hypertension or open-angle glaucoma [2]. It was formulated as eye drops or cream for applying skin. Redness, dry mouth and itchiness are the side effects associated with the use of brimonidine as eye drops. Headaches, redness and burning are the side effects possible while using it on skin [3].

Timolol (Fig. 1b) is a non-selective beta adrenergic blocker that can be taken on its own or in conjunction with other medications for the treatment of conditions that are characterized by elevated pressure within the eye, such as ocular hypertension and glaucoma [4]. When taken orally, timolol is effective in treating hypertension, reducing the risk of cardiovascular disease and alleviating the symptoms of migraines. While timolol gel was used to treat infantile hemangiomas, the eye drop formulation is utilized to treat open-angle and secondary glaucoma [5]. Cardiac arrhythmias and severe bronchospasms are the serious side effects associated with the use of timolol. Fainting, depression, confusion, congestive heart failure, impotence and worsening of Raynaud's syndrome are the less serious side effects associated with use of timolol [6].

Benzalkonium chloride (Fig. 1c) is a quaternary ammonium compound uses as phase transfer agent, cationic surfactant and bioactive agent [7]. It was used as preservative in the preparation of various pharmaceutical products eye, ear and nasal sprays or drops. It was also used in the preparation of wet wipes, hand sanitizers, shampoos, deodorants, cosmetics, soaps, throat lozenges, mouthwashes, spermicidal creams, skin antiseptics and wound wash sprays [8,9]. It has global signifi-



(a) Brimonidine **(b) Timolol** **(c) Benzalkonium chloride**

Fig. 1. Molecular structure of analytes in the study

cance and applicability as preservative in ophthalmic preparations but its shows significant toxicity and irritant properties. Hence, companies are focusing to prepare preservative free preparations or replace benzalkonium chloride with less harm preservative [10].

In literature, one UPLC method is reported for the analysis of brimonidine, timolol and benzalkonium chloride in ophthalmic drops [11]. Methods reported for the estimation of brimonidine and timolol in formulations using HPLC [12], UPLC [13], HPTLC [14] and UV-visible spectrophotometer [15]. One UPLC coupled with mass analyzer method was reported for impurity analysis of brimonidine and timolol [16]. Methods were available for the estimation of brimonidine and timolol in combination with dorzolamide [17] and latanoprost [18]. Based on the literature review, no HPLC method is reported for the quantification of brimonidine, timolol and benzalkonium chloride. Thus, in present work, a simple stability-indicating HPLC method is developed for the simultaneous quantification of brimonidine, timolol and benzalkonium chloride in the anti-glaucoma ophthalmic formulations.

EXPERIMENTAL

The API of brimonidine (99.02%), timolol (98.84%) and benzalkonium chloride (98.62%) were procured from Allergan India Pvt. Ltd., Bengaluru, India and its ophthalmic formulation solution with brand Combigan® (0.05 mg of benzalkonium chloride, 2 mg of brimonidine tartrate and 5 mg of timolol) were purchased from the local market. The HPLC grade methanol, acetonitrile and ultra-pure (Milli-Q®) were purchased from Merck Chemicals, India. Similarly, analytical reagent grade chemicals like hydrogen peroxide, sodium hydroxide, hydrochloric acid and buffer chemicals were also purchased from Merck chemicals, India.

Instrumental conditions: The study was conducted on Agilent (USA) 1100 HPLC instrument that comprises of G1311 Aquaternary pump for delivery of solvents, 0.1-1500 µL volume injectable auto-sampler with thermostat and UV detector (G 1314 A). Various configurations of stationary phases were used for the method development studies and the column eluents were integrated using Agilent chem-station software.

Standard solutions: An accurately weighed 25 mg of brimonidine, timolol and benzalkonium chloride were dissolved separately in a 25 mL clean and dry volumetric flask. Then 25 mL of methanol was added separately in each flask and sonicated the flasks for 2 min to dissolve the analytes completely in the solvent. Then the content was filtered through 0.2 µ membrane filter in a separate clean and dry flask separately and the final volume was made up to the mark with the same solvent. The brimonidine, timolol and benzalkonium chloride standard solution at a concentration of 1000 µg/mL was obtained separately. The combined standard solutions were prepared by accurately mixing equal volumes of individual known standard stock solutions in a separate flask and were used for method development and validation study [19].

Test solution: The Combigan® (0.05 mg of benzalkonium chloride, 2 mg of brimonidine tartrate and 5 mg of timolol) ophthalmic formulations were used for the preparation of sample solution. An accurately pipetted 1 mL of formulation pipetted in 10 mL volumetric flask containing 5 mL of methanol. The solution was sonicated for 2 min using ultrasonic bath sonicator and filtered through 0.2 µ membrane filter in to a clean and dry 10 mL volumetric flask. The final volume was made up to the mark using same diluent and the formulation stock solution at 200, 500 and 5 µg/mL of brimonidine, timolol and benzalkonium chloride, respectively. The formulation stock solution was further diluted to required concentration using the same diluent and the selected concentration solution was used for the quantification of brimonidine, timolol and benzalkonium chloride in formulation sample [20].

Method development: The systematic method development strategies [21] were applied for developing method for the simultaneous analysis of brimonidine, timolol and benzalkonium chloride. While developing the analytical method, the maximum absorbing wavelength for the detection of analytes was assessed using spectrophotometer. The iso-absorption wavelength of brimonidine, timolol and benzalkonium chloride was determined using spectrophotometer and the iso-absorption wavelength was fixed as detection wavelength during the development of HPLC method. During the initial method development steps, the mobile phase flow rate was fixed as 1.0 mL/min and after the completion of the development; the flow was further optimized in the range of 0.5 mL/min to 1.5 mL/min. The analytes in the study were polar in nature and the non-polar columns were utilized as stationary phases in the development of method. The high non-polar C_{18} columns of various brands and configurations were studied as stationary phase in the development study. The solvent ratio and its pH was finalized

by change in various ratios of the mobile phase with different pH ranges was studied.

In all the method development conditions studied, the standard solution containing 20, 50 and 0.5 µg/mL of brimonidine, timolol and benzalkonium chloride, respectively was injected and the chromatographic response was recorded. The peak area response, peak intensity, peak shape and the system suitability was summarized in all the studied conditions. The method conditions that produce best system suitability with high peak intensity and significantly no noise was considered as suitable conditions for the separation and analysis of brimonidine, timolol and benzalkonium chloride. These developed method conditions were further studied for method validation study.

Method validation: The standard solution containing brimonidine at 20 µg/mL, timolol at 50 µg/mL and benzalkonium chloride at 0.5 µg/mL was analyzed in the optimized method and the chromatographic response of resultant chromatograms was summarized for evaluating system suitability. The blank (diluent only), placebo solution prepared with commonly used formulation excipients was analyzed in the developed method for evaluating method specificity. A series of dilution of brimonidine, timolol and benzalkonium chloride were prepared in various concentration levels. The prepared dilutions were analyzed in the developed method and the peak area response of standard and both the impurities were tabulated separately. The calibration curve was constructed for brimonidine, timolol and benzalkonium chloride separately by taking the peak area response of analyte in *y*-axis and its concentration on *x*-axis. The correlation coefficient and the regression equation of standard brimonidine, timolol and benzalkonium chloride were derived from its corresponding calibration graphs.

The method accuracy was evaluated by performing the spiked recovery study and was performed at 50%, 100% and 150% spiked levels. The spiked level solution of brimonidine, timolol and benzalkonium chloride was spiked to 100% formulation solution and the recovery solution was analyzed in the optimized method. The peak area response of the recovery solution was compared with the calibration curve results in the same level and the % recovery of each analysis results and in each spiked level the % relative standard deviation (%RSD) was calculated. The % recovery of 98-102 and %RSD of < 2 was considered as acceptable.

The reproducibility of the method was evaluated in terms of precision and carried as intra-day and inter-day precision. In this method, the standard containing 20 µg/mL of brimonidine, 50 µg/mL of timolol and 0.5 µg/mL of benzalkonium chloride was assessed six times in one day for intra-day precision and 6 times in three consecutive days for inter-day precision. The peak area response of standard and both impurities was tabulated and the %RSD of the peak area response was calculated. The %RSD of less than 2 in both the precision studies for all the analytes was considered as the method was precise and repeatable.

The efficiency of the developed method that remains unaffected when there is a small change in the established method conditions as well as the change in analyte was assessed in ruggedness and robustness study. In ruggedness, the solution at precision level was prepared and analyzed by three different analysts and the peak area values were tabulated and%RSD of < 2 was acceptable. In robustness study, both positive and negative minor variations in the established method conditions made intentionally and the standard solution at precision level was analyzed in each changed condition. The % change in peak area of each analyte in each changed condition was determined and a value of < 2 was acceptable.

The smallest analyte concentration that can detect and quantify in the established method was considered as limit of detection and quantification, respectively. This information of the method confirms its sensitivity. The signal (s) to noise (n) ratio method was adopted for the evaluation of sensitivity.

The stability indicating nature of the method was assessed by performing stress degradation studies and the stress studies such as acidic, base, peroxide, thermal and UV light degradation studies was performed to the standard drug. An accurately weighed 50 mg of standard brimonidine, timolol and benzalkonium chloride was mixed separately with 50 mL of HCl (0.1 N), NaOH (0.0 N) and H_2O_2 (3%) in acid, base and peroxide degradation studies, respectively. The solutions were incubated for 24 h in dark, neutralized and then bring it to standard concentration prior to the analysis. The standard brimonidine, timolol and benzalkonium chloride was exposed to 60 ºC for 24 h in an air oven and UV light at 254 nm for 24 h in thermal and UV light degradation studies, respectively. Both these standard drugs after stress exposer were diluted to standard concentration prior to the analysis. All the stress exposed dilute solutions were evaluated in the established method and the chromatograms observed in each analysis were observed for confirming the acceptability of the method. The resultant chromatograms provides the number of stress degradation compounds generated as a results of stress exposer and the method applicability for the separation of stress degradation compounds was assessed. The peak area in each stress study was used for calculating the % degradation of brimonidine, timolol and benzalkonium chloride by comparing with unstressed peak area response in the developed method.

The developed method was applied for the separation, detection and quantification of brimonidine, timolol and benzalkonium chloride in formulation. The formulation sample solution prepared from Combigan® ophthalmic drops was assessed in the developed method. The peak area response was used to calculate the %content in the sample by comparing with corresponding standard calibration curve results.

RESULTS AND DISCUSSION

The individual UV absorption spectrum of brimonidine, timolol and benzalkonium chloride was measured in the scan range of 400-200 nm. The overlay wavelength scanning spectrum of individual wavelength scanning results confirms 227 nm as suitable wavelength for the detection of analytes. Hence, 227 nm was preliminary confirmed as suitable wavelength and was fixed as detector wavelength in the method development study.

Initially, Zodiac C_{18} (100 mm × 4.6 mm, 3 µm) column was selected as stationary phase, pH 5.0 acetate buffer and

acetonitrile in equal volumes was selected as mobile phase. The chromatogram identified in this condition (Fig. 2a) shows no clear separation of analytes. The baseline of the chromatogram throughout the run time was fluctuated and the separation was very poor with early retention time. This confirmed that 100 mm column was not suitable for the separation of analytes and hence further trail was proposed with increase in the length of the stationary phase.

The 250 mm Kromasil C_{18} (5 µm) column was utilized for the separation of analytes using pH 5.5 acetate buffer and methanol in in the ratio of 75:25 (v/v) as mobile phase. In this condition, the peaks corresponds to the analytes in the study were detected and identified. The peak height and the peak area response of the peaks corresponds to brimonidine and timolol was obderved to be very less whereas the peak area response of benzalkonium chloride peak was observed to be more. Baseline in the chromatogram was stabilized compared to previous conditions (Fig. 2b). Hence, further trail was continued with change in composition of mobile phase and stationary phase.

The method development was continued with Inertsil (250 mm × 4.6 mm, 5 µm) column with pH 5.5 phosphate buffer and methanol in 25:75 (v/v) as mobile phase. The chromatogram obtained in this trail (Fig. 2c) show three peaks corresponds to the analytes in the study. The peak area response of the peaks corresponds to timolol and benzalkonium chloride was observed to be very less with poor resolution. Whereas the peak area response of peak corresponds to brimonidine was observed to be very high with acceptable symmetry. This proved that the column in the study was not able to resolve the analytes and hence further trail was proposed with change in the column as well as the mobile phase composition.

Further method development was continued with Cosmosil C_{18} (250 × 4.6 mm; 5 µ id) column as stationary phase, pH 4.7 acetate buffer and methanol in 40:60 (v/v) was selected as mobile phase. The chromatogram observed in this condition (Fig. 2d) shows clear separation of peaks corresponds to brimonidine, timolol and benzalkonium chloride. The baseline throughout the run time was noticed to be stabilized and the peaks identified were little broad with high tail factors. The area response of the peak corresponds in this condition was improved than the previous trails performed. The system suitability of the resultant peaks was noticed to be not acceptable and hence the conditions were not suitable for the analysis.

The method development trails was continued with increase and decrease in the composition of buffer in mobile phase, flow rate of mobile phase and detector wavelength for achieving the best chromatographic separation with acceptable system

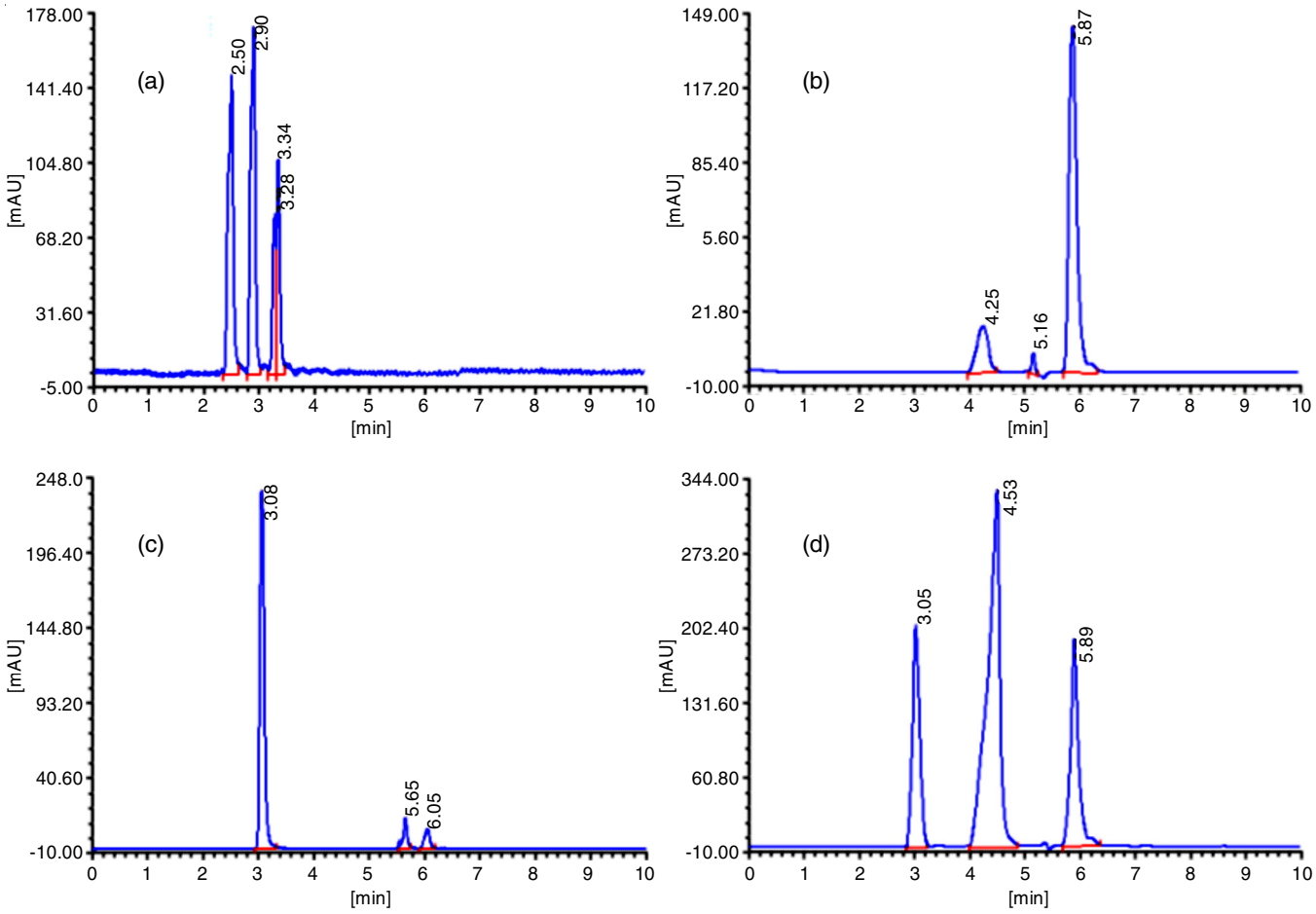


Fig. 2. Chromatograms observed during method development (a) No clear separation of analytes with high base line noise; (b and c) Poor peak area response of analytes with significantly less resolution of analytes; (d) Peaks corresponds to analytes was identified but it doesn't pass system suitability

TABLE-1
LINEARITY RESULTS

Brimonidine		Timolol		Benzalkonium chloride	
Conc. (µg/mL)	Peak area	Conc. (µg/mL)	Peak area	Conc. (µg/mL)	Peak area
5	77484.5	12.5	124585.3	0.125	19598.2
10	148582.3	25.0	279858.4	0.250	35362.8
15	234711.6	37.5	413263.8	0.375	54154.7
20	320651.9	50.0	551242.3	0.500	72857.1
25	402158.7	62.5	689265.1	0.625	91586.4
30	479858.1	75.0	845210.3	0.750	111853.7
35	562698.7	87.5	981545.3	0.875	130152.7
40	632157.4	100	1132209	1.000	151212.9

suitability. The separation was achieved on Cosmosil C_{18} (250 × 4.6 mm; 5 µ id) as stationary phase, pH 4.3 acetate buffer and methanol in 30:70 (v/v) as mobile phase at 1.0 mL/min and UV detection at 227 nm. In these optimized chromatographic conditions, clear separation of brimonidine, timolol and benzalkonium chloride was achieved with no additional detection of impurities or other co-eluting compounds. The analytes were identified at a retention time of at a retention time of 3.11 min for brimonidine, 4.86 min for timolol and 5.57 min for benzalkonium chloride whereas the chromatogram of blank doesn't show any chromatographic detections throughout the run time. This confirms that the established method was specific for the detection of brimonidine, timolol and benzalkonium chloride. The chromatogram of blank and standard observed in the developed method condition is represented in Fig. 3a-b, respectively.

The standard calibration curve solutions of brimonidine, timolol and benzalkonium chloride was prepared and analyzed in the optimized method. The high correlated calibration curve was attained in the analyte range of 5-40 µg/mL, 12.5-100 µg/mL and 0.125-1.0 µg/mL for brimonidine, timolol and benzalkonium chloride, respectively. The regression equation derived as $y = 16120x - 5403.5$ ($R^2 = 0.9994$), $y = 11424x - 15477$ ($R^2 = 0.9997$) and $y = 151150x - 1674.8$ ($R^2 = 0.9992$), respectively for brimonidine, timolol and benzalkonium chloride. The peak area results identified in the linearity study are represented in Table-1.

The standard solution containing 20 µg/mL of brimonidine, 50 µg/mL of timolol and 0.5 µg/mL of benzalkonium chloride was assessed in the optimized method for evaluating system suitability. The system suitability parameters of the chromatographic results were summarized and the method system suitability was assessed. As summarized in Table-2, the developed method passes the system suitability confirms the suitability of the developed method.

TABLE-2
SYSTEM SUITABILITY RESULTS

Parameter	Brimonidine	Timolol	Benzalkonium chloride
Concentration prepared	20 µg/mL	50 µg/mL	0.5 µg/mL
Retention time	3.11 min	4.86 min	5.57 min
Theo plate	4895	6715	9086
Tail factor	1.09	1.05	0.93
Resolution	–	8.29	5.03

The standard solution at 20 µg/mL of brimonidine, 50 µg/mL of timolol and 0.5 µg/mL of benzalkonium chloride was evaluated in precision and ruggedness study. The peak area response of each analyte was summarized in each study and the %RSD was calculated as 0.21, 0.10 and 0.34 in intraday precision, 0.30, 0.26 and 0.30 in interday precision and 0.31, 0.26 and 0.34 in ruggedness for brimonidine, timolol and benzalkonium chloride, respectively. The %RSD was achieved under the acceptable levels for all the analytes in each study proved that the method was precise and rugged.

The influence of the variations in the developed method conditions on the chromatographic response was assessed in robustness study. In robustness study, the composition of

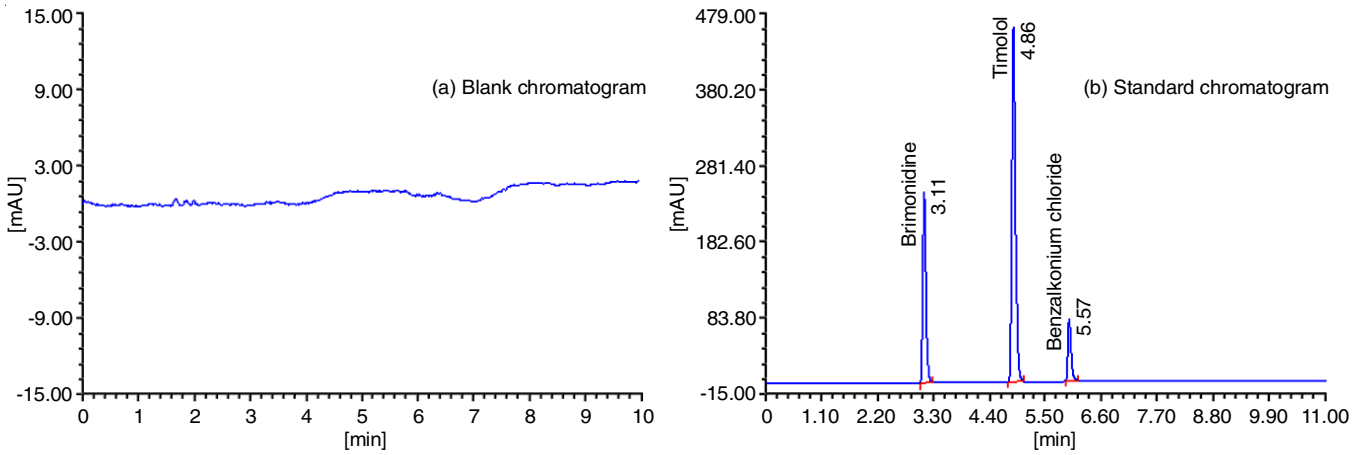


Fig. 3. System suitability chromatograms of brimonidine, timolol and benzalkonium chloride in the developed method

mobile phase was altered as 25:75 (MP 1) and 35:65 (MP 2) of buffer and methanol. The pH of buffer was altered as 4.2 (pH 1) and 4.4 (pH 2) as well as the detector wavelength of changed as 222 nm (WL 1) and 232 nm (WL 2). The % change in the peak area response of individual analyte was calculated and results reported under the acceptable levels. The system suitability of the individual analyte in each changed conditions is summarized (Table-3) and results reported under the acceptable levels confirms that the method was rugged.

The method accuracy was performed in spiked recovery study and the experiment was performed in the spiked levels of 50%, 100% and 150%. The standard solution in the linearity study *i.e.* 10 µg/mL of brimonidine, 25 µg/mL of timolol and 0.25 µg/mL of benzalkonium chloride was considered as target concentration in spiked recovery study. The solutions were evaluated in the optimized method and the peak area response was compared with the standard calibration results in the same level. The % recovery for each injection and the %RSD in each spiked level was calculated. The % recovery was observed to be with in the acceptable levels of 98-102% and the %RSD was observed to be less than 2 in each spiked level (Table-4) confirmed the method accuracy.

The s/n approach was adopted for the evaluation of LOD and LOQ of method optimized for analyzing brimonidine, timolol and benzalkonium chloride. The LOD was determined as 0.5, 1.25 and 0.0125 µg/mL, respectively for brimonidine, timolol and benzalkonium chloride. Based on LOD, the LOQ was calculated as 1.650, 4.125 and 0.041 µg/mL, respectively for brimonidine, timolol and benzalkonium chloride. This confirms that the method can effectively detect the impurities up to very low concentrations for all the analytes.

The method was evaluated for its applicability for the separation and analysis of various compounds generated due to stress degradation of brimonidine, timolol and benzalkonium chloride. The standard drug was exposed to various stress conditions and then the stressed sample was evaluated in the developed method. The resultant chromatograms (Fig. 4) and its results were analyzed for the evaluation of its applicability for the separation of stress degradants. In acid degradation study, three additional compounds were identified at 1.03, 3.60, 4.38 and 7.25 min with % degradation of 5.24%, 6.17% and 4.19%, respectively for brimonidine, timolol and benzalkonium chloride. Very high % degradation of analytes was noticed in UV light degradation study. In this, the % degradation was calculated as 7.11, 6.85 and 5.76%, respectively for brimonidine, timolol and benzalkonium chloride. Four additional degradation compounds were effectively resolved from the analytes and were identified at 1.23, 2.61, 3.63 and 6.87 min. In base

TABLE-3
ROBUSTNESS RESULTS

Compound	Change	Peak area	Change (%)	Plate count	Tail factor	Resolution
Brimonidine	MP 1	323736.9	0.96	4812	1.09	–
	MP 2	323657.3	0.94	4882	1.08	–
	pH 1	319011.5	0.51	4791	1.09	–
	pH 2	315476.4	1.61	4932	1.05	–
	WL 1	317368.7	1.02	4685	1.08	–
	WL 2	316855.3	1.18	4899	1.06	–
Timolol	MP 1	554629.8	0.61	6708	1.05	8.28
	MP 2	555668.9	0.80	6795	1.05	8.24
	pH 1	554806.9	0.65	6825	1.03	8.22
	pH 2	557115.3	1.07	6715	1.06	8.26
	WL 1	555628.2	0.80	6654	1.04	8.27
	WL 2	555041.7	0.69	6905	1.04	8.28
Benzalkonium chloride	MP 1	71832.9	1.41	9124	0.93	5.09
	MP 2	74037.7	1.62	8969	0.94	5.11
	pH 1	72822.4	0.05	9145	0.94	5.02
	pH 2	74134.9	1.75	9274	0.95	5.08
	WL 1	73926.9	1.47	9104	0.92	5.01
	WL 2	73430.1	0.79	9056	0.93	5.12

TABLE-4
RECOVERY RESULTS

Compound	Recovery level (%)	Concentration prepared (µg/mL)	Amount found (n = 3) Mean ± SD	% Recovered (n = 3) Mean ± SD	%RSD of recovery
Brimonidine	50	15	14.86 ± 0.080	99.08 ± 0.533	0.54
	100	20	19.74 ± 0.078	98.69 ± 0.391	0.40
	150	25	25.07 ± 0.152	100.27 ± 0.607	0.61
Timolol	50	37.5	37.07 ± 0.169	98.86 ± 0.450	0.45
	100	50	49.74 ± 0.128	99.47 ± 0.255	0.26
	150	62.5	62.80 ± 0.257	100.48 ± 0.411	0.41
Benzalkonium chloride	50	0.375	0.37 ± 0.002	98.51 ± 0.401	0.41
	100	0.5	0.49 ± 0.003	98.89 ± 0.645	0.65
	150	0.625	0.62 ± 0.005	98.98 ± 0.728	0.74

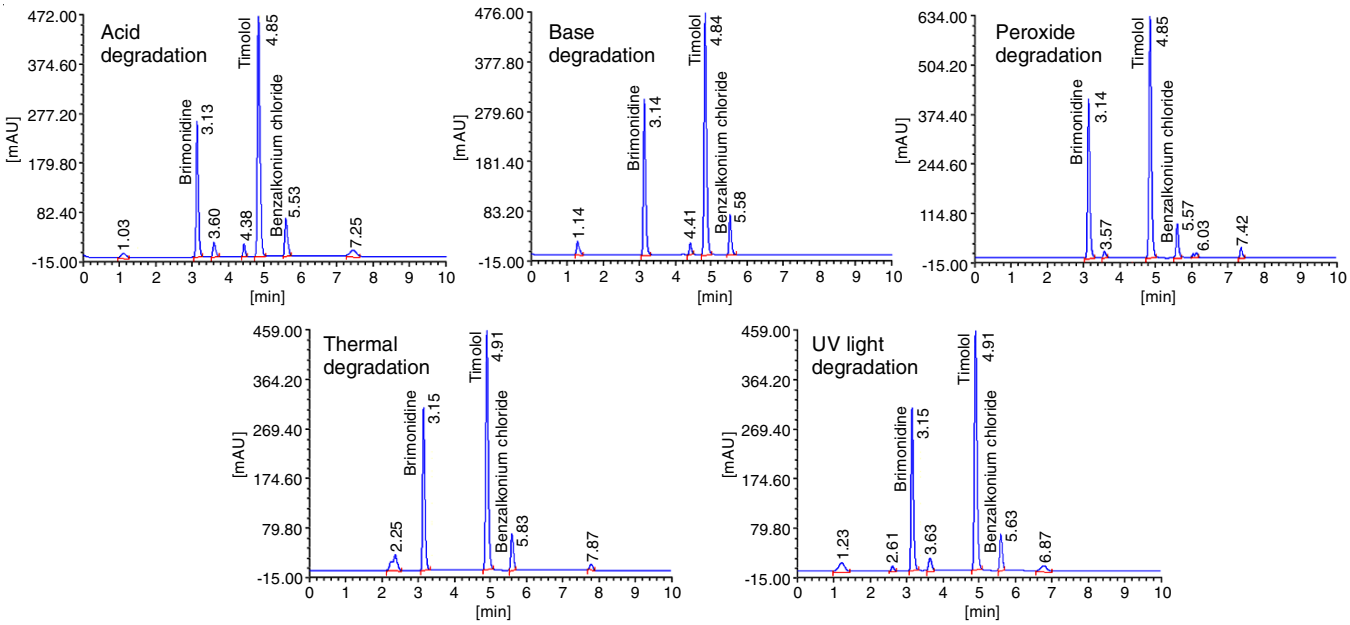


Fig. 4. Forced degradation chromatograms

and thermal degradation study, two additional degradation compounds were noticed whereas three additional compounds were observed in peroxide degradation study. In all the stressed conditions performed, the peaks correspond to brimonidine, timolol and benzalkonium chloride were detected and the retention time of analytes were in good correlation with the standard. The % degradation was noticed to be less in all the stressed conditions for all the analytes and the method can effectively resolve the stress degradants effectively proved that the method was stable.

The analytical method optimized in the study was applied for its applicability for the estimation of brimonidine, timolol and benzalkonium chloride in ophthalmic drops. The formulation solution prepared using Combigan® was used for the formulation assay study. The resultant chromatogram show clear identification and resolution of brimonidine, timolol and benzalkonium chloride. The assay was observed to be 98.92%, 99.36% and 98.46% for brimonidine, timolol and benzalkonium chloride respectively. In the chromatogram (Fig. 5), there is no detection of impurities and there is no detection of additional compounds as well as the formulation excipients. This confirmed that the method was significantly used for the evaluation of brimonidine, timolol and benzalkonium chloride in the ophthalmic dosage forms.

Conclusion

A simple and novel stability indicating analytical RP-HPLC method was optimized for separation and quantification of brimonidine, timolol and benzalkonium chloride in ophthalmic formulations. The method reports very sensitive detection limit of 0.5, 1.25 and 0.0125 µg/mL, respectively for brimonidine, timolol and benzalkonium chloride confirmed that the method can detect the analytes at very low levels. The other validation parameters such as specificity, system suitability, accuracy/recovery, repeatability and reproducibility results were under the acceptable level. The method can efficiently resolve, detect

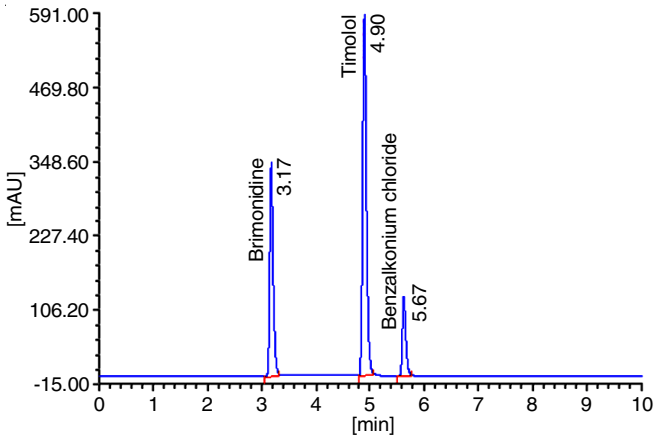


Fig. 5. Formulation chromatogram

and quantify unknown stress degradation products along with the analytes. Based on the obtained validation results and method application studies, it can be concluded that the method can effectively utilized for analyzing brimonidine, timolol and benzalkonium chloride in stress samples, bulk drug as well as ophthalmic formulations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. L.B. Cantor and J. Burke, *Expert Opin. Investig. Drugs*, **6**, 1063 (1997); https://doi.org/10.1517/13543784.6.8.1063
2. J.C. Adkins and J.A. Balfour, *Drugs Aging*, **12**, 225 (1998); https://doi.org/10.2165/00002512-199812030-00005
3. C.B. Toris, C.B. Camras and M.E. Yablonski, *Am. J. Ophthalmol.*, **128**, 8 (1999); https://doi.org/10.1016/S0002-9394(99)00076-8
4. M. Volotinen, M. Turpeinen, A. Tolonen, J. Uusitalo, J. Mäenpää and O. Pelkonen, *Drug Metab. Dispos.*, **35**, 1135 (2007); https://doi.org/10.1124/dmd.106.012906

5. T. Atkin, S. Comai and G. Gobbi, *Pharmacol. Rev.*, **70**, 197 (2018); https://doi.org/10.1124/pr.117.014381
6. J.H. Liu, D.F. Kripke and R.N. Weinreb, *Am. J. Ophthalmol.*, **138**, 389 (2004); https://doi.org/10.1016/j.ajo.2004.04.022
7. B.M.P. Pereira and I. Tagkopoulos, *Appl. Environ. Microbiol.*, **85**, e00377 (2019); https://doi.org/10.1128/AEM.00377-19
8. S.M. Choi, T.H. Roh, D.S. Lim, S. Kacew, H.S. Kim and B.M. Lee, *J. Toxicol. Environ. Health B Crit. Rev.*, **21**, 8 (2018); https://doi.org/10.1080/10937404.2017.1408552
9. O. Condell, C. Iversen, S. Cooney, K.A. Power, C. Walsh, C. Burgess and S. Fanning, *Appl. Environ. Microbiol.*, **78**, 3087 (2012); https://doi.org/10.1128/AEM.07534-11
10. M.H. Goldstein, F.Q. Silva, N. Blender, T. Tran and S. Vantipalli, *Eye*, **36**, 361 (2022); https://doi.org/10.1038/s41433-021-01668-x
11. H.M. Hafez, A.A. Elshanawany, L.M. Abdelaziz and M.S. Mohram, *Eurasian J. Anal. Chem.*, **10**, 46 (2015).
12. H.M. Hafez, A.A. Elshanawane, L.M. Abdelaziz and M.S. Mohram, *J Appl. Pharm.*, **6**, 398 (2014).
13. E. Büker and E. Dinç, *J. Chromatogr. Sci.*, **55**, 154 (2017); https://doi.org/10.1093/chromsci/bmw160
14. M.C. Damle and A. Zirange, *Int. J. Res. Culture Soc.*, **2(7)**, 1 (2018).
15. M.A. Mukthinuthalapati, S. Malineni and S.V.S. Vellanki, *J. Anal. Pharm. Res.*, **4**, 14 (2017).
16. S. Kondra, B. Akula Thukaram, D. Gowrisankar, P.A. Krishnamanjari, H.S.N. Raju Dantuluri and S. Maganti, *Ann. Pharm. Fr.*, **79**, 617 (2021); https://doi.org/10.1016/j.pharma.2021.02.006
17. J. Venkateswaran, M. Sundarapandian and S. Venkataraman, *Asian J. Pharm.*, **11**, S588 (2017).
18. M. Walash and R. El-Shaheny, *Yao Wu Shi Pin Fen Xi*, **24**, 441 (2016); https://doi.org/10.1016/j.jfda.2015.11.006
19. B.B. Kasimala, V.R. Anna and U.R. Mallu, *Indian Drugs*, **55**, 41 (2018).
20. U.R. Mallu, V.R. Anna and B.B. Kasimala, *Turk. J. Pharm. Sci.*, **16**, 457 (2019); https://doi.org/10.4274/tjps.galenos.2018.34635
21. ICH Expert Working Group, ICH harmonised tripartite guideline–Validation of Analytical Procedures Text and Methodology: Q2 (R1). In Geneva: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2005).