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Design of Experiment Utilization to Develop a Simple and Robust RP-UPLC Method for Stability Indicating Method of anti glaucoma Ophthalmic Drops

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Abstract

Brimonidine Tartrate and Timolol Maleate are used in treatment of glaucoma by decreasing intra ocular pressure. It is necessary to establish a Validated UPLC method for the assay of Brimonidine Tartrate and Timolol Maleate as well as Benzalkonium Chloride in eye drops. Using of DOE techniques provides more information about different factors affects system suitability of new method. DOE led to highly robust methods through creating design space. The method was performed on Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) and the mobile phase consisted of Methanol and 1- Decane sulphonic acid sodium (pH 3.0, 0.01M) which pumped at a flow rate 0.6 mL min^{-1} at 50 °C in gradient manner. 10 µL of drugs sample solutions were monitored at wavelengths 320 nm. Brimonidine Tartrate, Timolol Maleate and Combigan® ED were stressed under different conditions in forced degradation studies. Degradation products had a good resolution with main drugs and Benzalkonium Chloride. The proposed method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to ICH.

Keywords:

Brimonidine Tartrate; Timolol Maleate; Benzalkonium Chloride ,UPLC; Stability Indicating Method; Design of Experiment

1. Introduction

Glaucoma describes a group of disorders characterized by a loss of visual field associated with cupping of the optic disc and optic nerve damage. It is generally associated with raised intra-ocular pressure. Forms of glaucoma are primary open-angle glaucoma and primary angle closure glaucoma.

Drugs that reduce intra-ocular pressure by different mechanisms are used for managing glaucoma. A topical beta-blocker or a prostaglandin analogue is usually the drug of first choice. It may be necessary to combine these drugs or add others, such as miotics, carbonic anhydrase inhibitors or *sympathomimetics* to control intra-ocular pressure [1].

Brimonidine is 5-Bromo-N-(4, 5-dihydro-1H-imidazol-2-yl) quinoxalin-6-amine [2, 3] as shown in Fig 1, it is a selective alpha -adrenoceptor agonist. It is licensed for the reduction of intra-ocular pressure in open-angle glaucoma or ocular hypertension [1].

Timolol Maleate is (2S)-1-[(1, 1- dimethyl ethyl) amino]-3-[[4-(morpholin-4-yl)-1, 2, 5-thiadiazol-3-yl] oxy] propan-2-ol (Z) - butenedioate [2, 3] as shown in Fig 1, it is a non-selective beta blocker. It is reported to lack intrinsic sympathomimetic and membrane-

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stabilizing activity. Timolol Maleate is used in the management of glaucoma and hypertension [1].

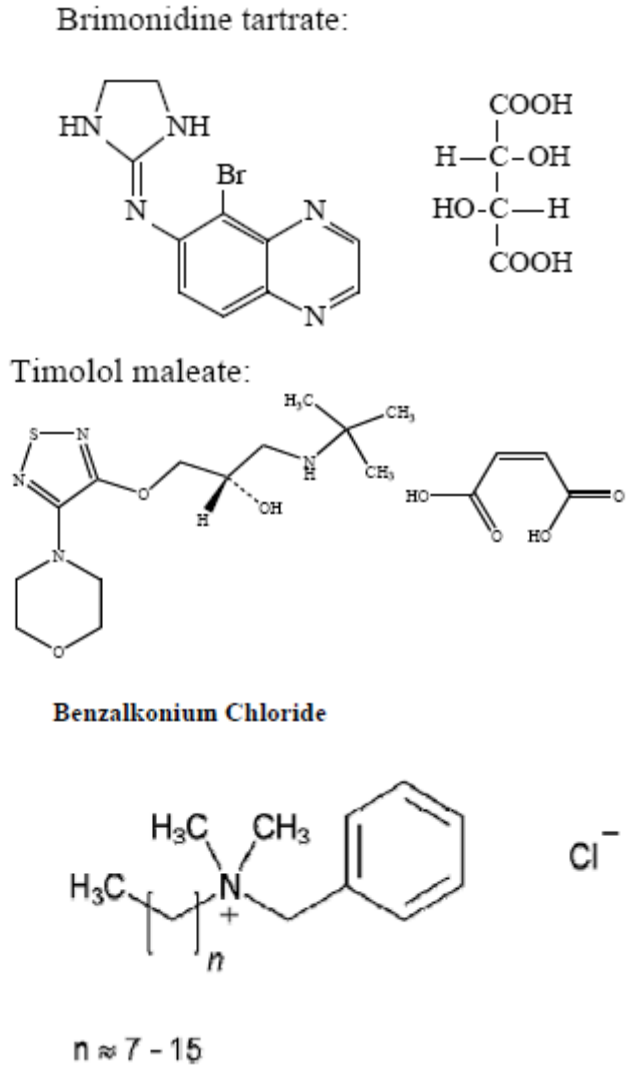


Fig 1: Structures of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride

Combigan® eye drop contains Timolol Maleate equivalent to 0.5% of Timolol and 0.2% of Brimonidine Tartarate. It is instilled twice daily to reduce raised intra-ocular pressure in open-angle glaucoma and ocular hypertension.

Benzalkonium chloride (BAK) is a bacteriostatic agent used in the pharmaceutical industry as a preservative. The most important type of BAK is that contains alkyl substituents C12 and C14 with the quaternary ammonium salt [4].

In view of the above, control of Benzalkonium chloride identity and content in pharmaceutical preparations is necessary. In addition, the analytic method should enable to determine both total BAK content.

BP 2014 estimated Timolol Maleate and Benzalkonium chloride potentiometrically [2, 3]. USP 2014 described RP-HPLC for determination Timolol Maleate and Benzalkonium chloride individually [3]. Brimonidine is not official in USP 2014 and BP 2014 [2, 3].

Few methods were reported for estimation of Timolol Maleate and Brimonidine such as spectroscopy [5], HPTLC [6] and HPLC methods [7, 8].

Literature review reveals that there is not any analytical method was established for simultaneous of Timolol Maleate, Brimonidine and Benzalkonium chloride as the main eye drop preservative. No stability indicating method for these drugs was found in literature. The purpose of this study was to develop a robust stability indicating method for Brimonidine Tartrate, Timolol Maleate as well as Benzalkonium chloride in ophthalmic drops.

Developed method will utilize design of experiments (DOE) technique because it has some advantages include that many factors can be studied simultaneously, it achieves structured analysis of main effect, interactions and noise, it is efficient in terms of the amount of information obtained given the number of runs made and it has rigorous statistical methods for the analysis.

2. Experimental

2.1. Instrumentation

Analysis was performed on a chromatographic system of Waters Acquity UPLC equipped with a binary solvent delivery pump, an auto sampler and connected to TUV detector. The system equipped by Empower PC program (Waters, Milford, USA). The chromatographic separation was achieved on a Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) column.

Method development and method modeling were performed using MODDE 9 Trial version 2014 software

2.2. Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. 1- Decane sulphonic acid sodium and ortho phosphoric acid were supplied by Merck (Darmstadt, Germany). Methanol HPLC grade was supplied by Fischer scientific (U.K.). Distilled water was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA).

Timolol Maleate, Brimonidine Tartrate and *Benzalkonium Chloride* working standard powders were kindly supplied by Egyptian international pharmaceutical industries company (EIPICO) (10^{th} of Ramadan, Egypt) and were used without further purification.

2.3. Pharmaceutical preparation

Combigan® eye drops (Allergan, USA) contain Brimonidine Tartrate 0.2%, Timolol (as Maleate) 0.5% and Benzalkonium Chloride 0.005% B.NO: E64005.

2.4. Chromatographic condition

10 µL of drugs sample solutions were monitored at a fixed wavelength 320.0 nm. Liquid chromatography was performed on a Phenomenex Kinetex C18 (50x4.6 mm, 2.6 µm) column and the mobile phase was consisted of Methanol and 1- Decane sulphonic acid sodium (pH 3.0, 0.01M) which pumped at a flow rate 0.6 mL min^{-1} at 50 °C in a gradient manner as shown in Table1.

1- Decane sulphonic acid sodium (0.01 mol L^{-1}) was prepared by dissolving 2.44 g of 1- Decane sulphonic acid sodium in approximately 1000 mL distilled water. The pH was adjusted to 3.0 with ortho phosphoric acid. The mobile phase was filtered through a 0.45 µm Nylon membrane filter (Millipore, Milford, MA, USA) under vacuum and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage.

Table 1: Time Table of Validated Gradient Method

Time minutes	Flow Rate mL minute^{-1}	Methanol %	Buffer %	Wavelengths nm
0	0.6	50	50	320
3.5	0.6	80	20	320
4.0	0.6	80	20	210
6	0.6	80	20	210
6.5	0.6	50	50	210
8	0.6	50	50	210

2.5. Preparation of stock standard solutions

Stock standard solutions contain 2, 6.8 and 0.05 mgmL^{-1} of Brimonidine Tartrate , Timolol Maleate (equivalent to 5 mgmL^{-1} of Timolol) and Benzalkonium Chloride respectively were prepared by dissolving 200, 680 and 5 mg of each in distilled water in 100 mL volumetric flask respectively. It was then sonicated for 10 minutes and the final volume of solutions was made up to 100 mL with distilled water to get stock standard solutions.

2.6. Preparation of calibration plot (working standard solutions)

To construct calibration plots, the stock standard solutions were diluted with distilled water to prepare working standard solutions in the concentration ranges (1-100, 2.25-225 and 1-10 µgmL^{-1}) of Brimonidine Tartrate, Timolol and Benzalkonium Chloride respectively. Each solution (n=10) was injected in triplicate and chromatographed under the mentioned conditions above. Linear relationships were obtained when average drug standard peak area were plotted against the corresponding concentrations for each drug. Regression equation was computed.

2.7. Sample preparation

2 mL of Combigan® ED were taken into 50 mL volumetric flask and completed with distilled water. Test solutions were analyzed under optimized chromatographic conditions.

2.8. Forced degradation of Brimonidine Tartrate and Timolol Maleate

To determine the proposed method as a stability-indicating method for Brimonidine Tartrate, Timolol Maleate and Combigan® ED were stressed under different conditions in forced degradation studies. Stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED used to forced degradation studies - were prepared by dissolving it in distilled water [9, 10].

2.8.1. Acidic degradation

Hydrochloric acid (HCl) (1 mol L^{-1}, 10 mL) was added to 10 mL prepared stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED respectively. These solutions were separately heated at 75C° for 6 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) were then transferred to 10 mL volumetric flasks, neutralized by addition of 1mL of 1 M NaOH and diluted to final volume with distilled water [9, 10].

2.8.2. Alkaline degradation

Sodium hydroxide (NaOH) (1 mol L^{-1}, 10 mL) was added to 10 mL prepared stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) were then transferred to 10 mL volumetric

flasks, neutralized by addition of 1mL of 1 mol L^{-1} HCl, and diluted to final volume with distilled water [9, 10].

2.8.3. Oxidation

Hydrogen peroxide (H_2O_2; 10%, v/v, 10 mL) was added to 10 mL prepared stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light). The solutions (2 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water [9, 10].

2.8.4. Neutral degradation (Thermal degradation)

10 mL of distilled water was added to 10 mL prepared stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED respectively. These solutions were separately heated at 75C° for 4 hours in the dark (to exclude the possible degradative effect of light) to study the effect of thermal stress. The experiment was also performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of distilled water. The experiment was performed in the dark to exclude the possible degradative effect of light The solutions (1 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water [9, 10].

2.8.5. Photo stability

10 mL of distilled water was added to 10 mL prepared stock solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED respectively. These solutions were separately exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours $meter^{-2}$. The experiment was also performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of distilled water. The solutions (1 mL) obtained were then transferred to 10 mL volumetric flasks and diluted to final volume with distilled water [9, 10].

2.9. Optimization of chromatographic condition

The ability of a chromatographic method to successfully separate, identify, and quantitate species was determined by several factors which are in the control of the experimenter. Attempting to discover the importance of these factors with respect to the response, design of experiments (DOE) will be utilized to give a powerful suite of statistical methodology which is capable of estimating the effects of each factor in combination as well as alone.

Few trials were carried to determine Brimonidine Tartrate and Timolol Maleate in Combigan® ED simultaneously with Benzalkonium Chloride. Some problems were noticed during early trials using UPLC. These problems are the interference between Maleic acid and Brimonidine or Brimonidine and Timolol peaks and long retention time of Benzalkonium Chloride.

Firstly, maximum absorption wavelengths for Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride were scanned from 400-200 nm under UV as shown in Fig 2. It was found that all drugs have high absorption at the universal wavelength 210 nm so, it was selected for DOE trials because it is the sole maximum wavelength of Benzalkonium Chloride which characterized by low concentration in Combigan® eye drop and weak absorptivity under UV.

Low concentration of used buffers (0.01M or 0.5%) is adequate for most reversed phase applications. This concentration is also low enough to precipitation problems when significant amounts of organic modifiers are used in the mobile phase [11].

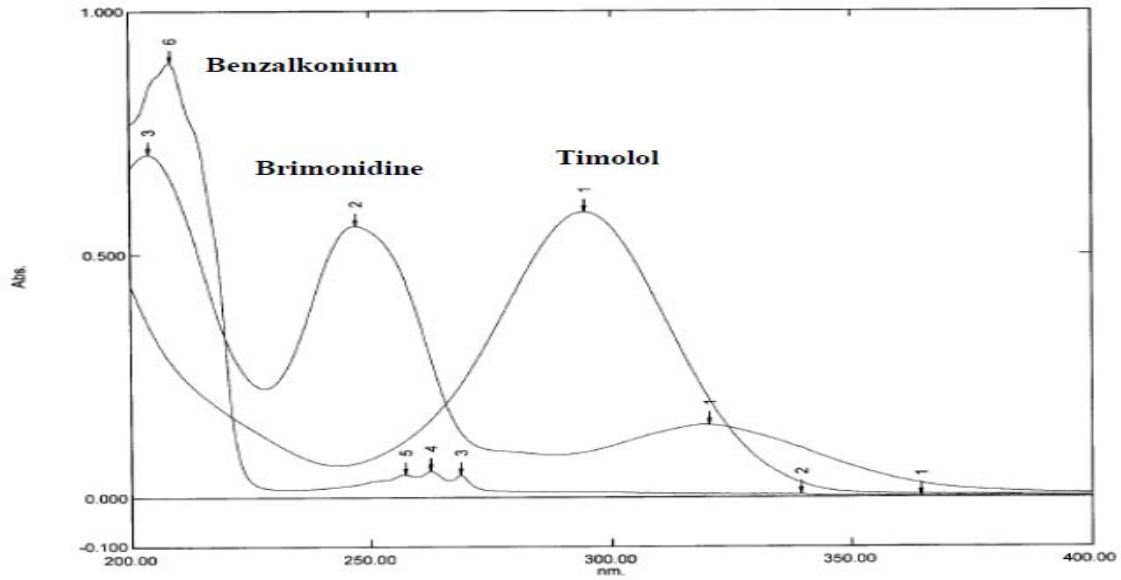


Fig 2: Typical UV spectrum of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride

2.10. Development strategy

Our innovative development strategy follows design of experiment (DOE) principles and can be divided into the five steps: (1) Definition of method goals, (2) risk assessment, (3) design of experiments (4) Design Space, (5) working point selection and verification [12].

2.10.1. Definition of method goals (critical quality attribute)

The primary goal of developing an UPLC stability indicating method is generally to separate the drugs from their impurities (resolution Rs > 2.0) that may impact the quality of the pharmaceutical formulation.

From the general equation $Rs = 0.25*N^{1/2}[(\alpha-1/\alpha)]\ (k/1 + k)$, it is obvious that the selectivity parameter has the greatest impact on resolution. Selectivity can be changed by modification of the mobile phase composition, column chemistry and temperature [13].

Other factors such as the need for short analysis times (<10 min) are also considered as critical quality attributes (CQAs) as shown in Table 2. Crucial for the design of experiment approach is to create a visual "Design Space" in which the method is robust.

2.10.2. Risk assessment (critical method parameters)

In an early risk assessment the critical parameters should be identified. That could be method factors which may affect extraction of the compounds of interest in sample preparation (e.g. extraction method, extraction time, extraction solvent) [14] as well as settings in the instrumental analysis.

For example the UV spectra of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride were evaluated to select the detection wavelength as mentioned before. Flow rate of mobile phase was 0.6 mLmin-1 and column temperature was 50 C°. Further on the critically influential separation parameters such as stationary phases, type and percent of the eluent A, type and pH of the eluent B were identified and considered as critical method parameters (CMPs) as shown in Table 2 [15].

Table 2: critical method parameters (CMP), critical quality attribute (CQA) and quality method target profile (QMTP)

CMP	Range for each parameter used for QbD		CQA	QMTP
	Low	High		Targeted QTMP
1- Stationary phases	Kinetex C18 (50x4.6 mm, 2.6µm)	SiliaBond C18 (50x 2.1 mm, 1.8µm)	Resolution between Maleic acid and Brimonidine Tartrate (Res 1)	Resolution should be more than 2.0
			Resolution between Brimonidine Tartrate and Timolol Maleate (Res 2)	Resolution should be more than 2.0
2- Type of the eluent A	Methanol (MeOH)	Acetonitrile (Acet)	Retention time of Benzalkonium chloride (time)	Retention time should be less than 10 min
3- Percent of the eluent A	20:80 in 4 min	50:80 in 4 min	Theoretical plates (plate)	1500 - 2500
4- Type of the eluent B	0.5% Triethylamine (TEA)	0.5% Perchloric acid (Perchlor)	Symmetry factor (sym)	0.72 - 1.0
	0.5% Ortho phaosphoric acid (phosphate)	0.02M Decane sulphonic acid (DEC)	Capacity factor (K)	2 - 5
5- pH of the eluent B	2.5	5.5		

2.10.3. Design of experiments (screening and optimization)

As the result of the risk assessment and identification of CMPs, the five parameters were screened and optimized using screening design in MODDE software. After all data (CMPs and CQAs) had entered, MODDE software set 19 chromatographic runs to be carried out as shown in Table 3.

In this approach the chromatograms obtained by two stationary phases, two type of eluent A, three eluent A compositions, four type of eluent B and 3 pH degrees of eluent B were necessary in order to 4D contour plots (resolution maps) and further on a 4D-sweet spot model (design space) of the critical resolution by using MODDE software as shown in Fig 3 and 4. The ranges between these factors were large enough to induce peak movements to discover hidden peaks and changes in the selectivity as a result of movement of peaks can be studied [16].

2.10.4. Design Space and experimental results evaluation

Design space is the region in which changes to the method parameters will not significantly affect the results. After processing of 19 experimental trials, the resolution between 5 peaks of interest (Maleic acid, Brimonidine, Timolol and Benzalkonium Chloride (BKC1 and BKC2)) and retention time of BKC2 were determined in each chromatogram and input to MODDE software as shown in Table 3.

These experimental results were evaluated using stringent criteria (quality method target profile, QMTP) as shown in Table 2 to create the design space.

Design space (shown in green color in Fig 4 a-d) allows alteration of the position of the “working point” without the need for a new validation and a high flexibility in the HPLC/UHPLC laboratory.

Table 3: Experimental design (19 runs) obtained by MODDE software for developed method of Combigan® ED

Exp No	Exp Name	Run Order	Incl/ Excl	Buffer Type	Org Solv Type	Buffer pH	Org Solv %	Column Type	Res1	Res 2	K	sym	Time	Plate
1	N1	1	Incl	Phos	Acet	2.5	20	SiliaBond	0	1.65	1.44	0.75	5.3	154
2	N2	7	Incl	TEA	Acet	5.5	20	SiliaBond	0	1.4	1.37	0.65	6.8	105
3	N3	17	Incl	DEC	Acet	5.5	20	SiliaBond	7.9	2.7	0.75	0.65	7	181
4	N4	9	Incl	Perch	Acet	2.5	50	SiliaBond	0	0	1.1	0.6	4	83
5	N5	10	Incl	Phos	MeoH	5.5	50	SiliaBond	0	0.75	1.2	0.58	6	84
6	N6	14	Incl	TEA	MeoH	2.5	50	SiliaBond	0	0	1.48	0.7	5	134
7	N7	18	Incl	DEC	MeoH	2.5	20	SiliaBond	2.2	3.5	0.99	0.7	6	179
8	N8	19	Incl	Perch	MeoH	5.5	50	SiliaBond	0.8	1.45	1.13	0.7	5.4	201
9	N9	8	Incl	Phos	Acet	2.5	50	Kinetex	0	0	4.6	0.58	6	1392
10	N10	6	Incl	TEA	Acet	5.5	50	Kinetex	0	0.28	4.5	0.55	7	1004
11	N11	11	Incl	DEC	Acet	2.5	50	Kinetex	0.7	0.8	4.3	0.75	7	1483
12	N12	12	Incl	Perch	Acet	5.5	20	Kinetex	3.7	4.3	4.59	0.72	8.2	1779
13	N13	13	Incl	Phos	MeoH	5.5	20	Kinetex	1.24	3.6	5.07	0.72	7.9	1872
14	N14	15	Incl	TEA	MeoH	2.5	20	Kinetex	1.94	6.5	6.5	0.75	9	2852
15	N15	16	Incl	DEC	MeoH	5.5	50	Kinetex	4	6.5	4.5	0.73	11	1423
16	N16	4	Incl	Perch	MeoH	2.5	20	Kinetex	3.95	6.25	5.81	0.76	9	2459
17	N17	2	Incl	Perch	MeoH	4	35	Kinetex	1.3	4.85	5.2	0.8	9	1781
18	N18	3	Incl	Perch	MeoH	4	35	Kinetex	1.3	4.8	5.2	0.8	9	1835
19	N19	5	Incl	Perch	MeoH	4	35	Kinetex	1.35	4.85	5.2	0.8	9	1824

The color code in these resolution maps represents the value of the critical resolution, with “green” colors show large resolution values (it conforms quality target method profile (QTMP) as shown in Table 2), yellow, light and dark “blue” colors show low resolution values (it does not conform QTMP) [16].

2.10.5. Working point selection and verification

From the previously constructed design space, the working point was selected by visual examination looking for the highest critical resolution (Rs, crit), best robustness of the method and shorter analysis time.

At this point, small changes of CMPs as well as flow rate and dwell volume have no negative influence on the separation of all peaks. This working point was found in the sweet spot plot as mentioned under chromatographic conditions except detector wavelength (320 nm).

2.10.5.1. Selection of proper detector wavelength

Although 210 nm was set for DOE trials as a constant factor, a problem in linearity and precision appeared during method verification and validation. Selection of test concentration of studied drugs was subjected to some considerations such as low concentration of benzalkonium chloride, its low absorptivity and matching selected concentration with the drug concentration ratio in Combigan® ED.

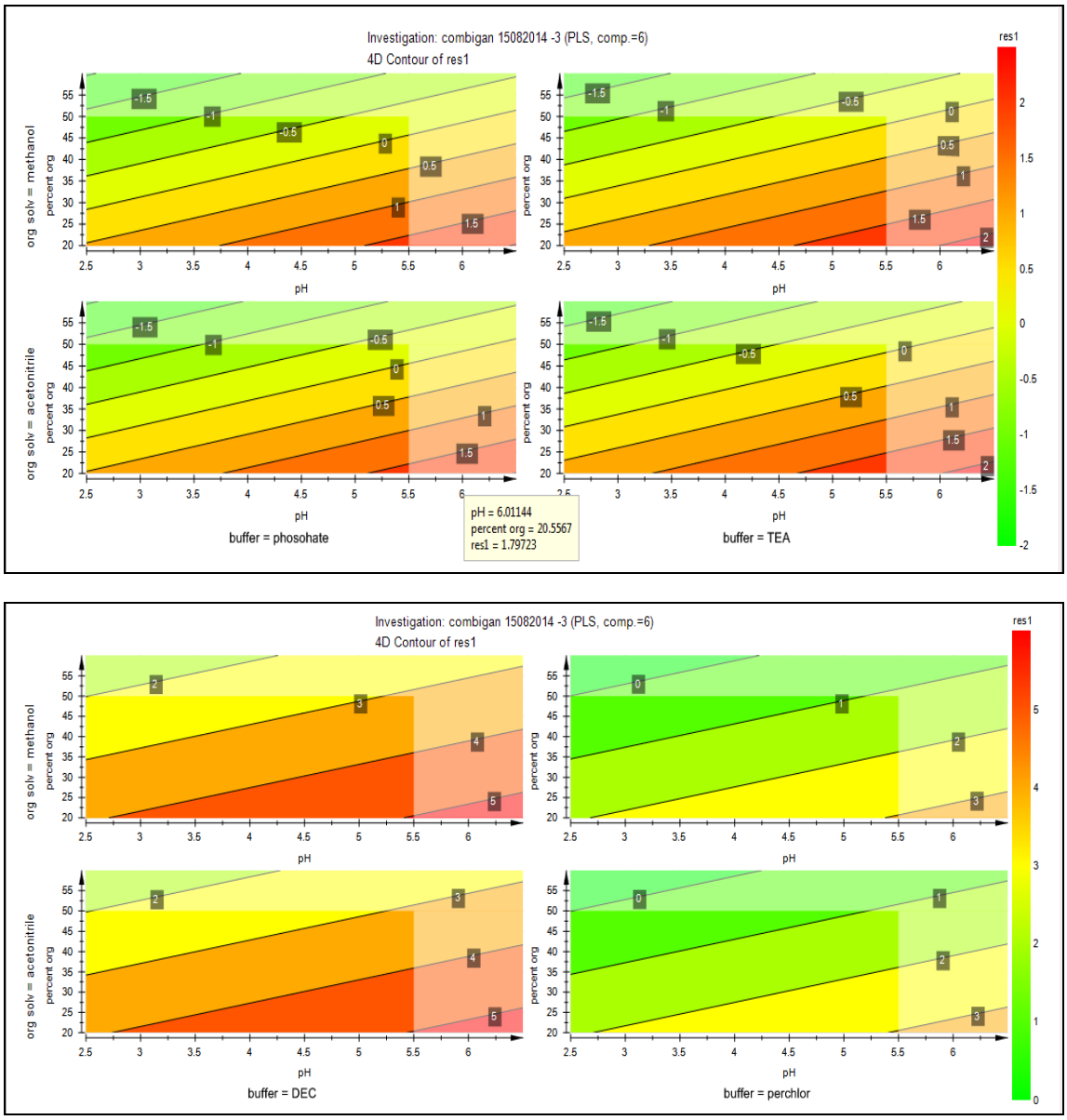
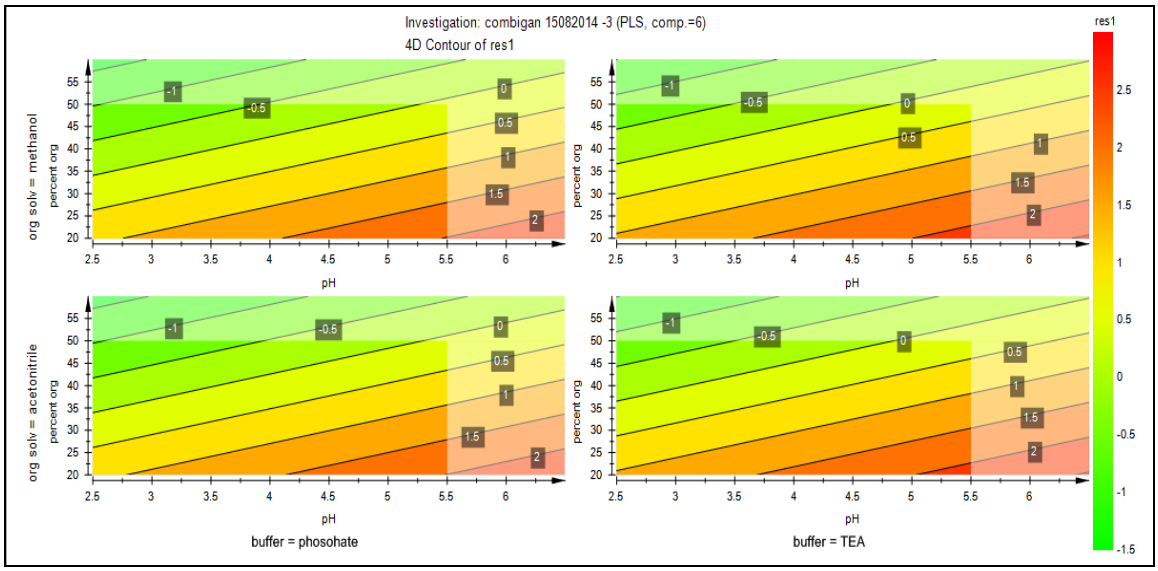


Fig 3a-b: 4D Contour plot (Resolution Map) for resolution between Maleic acid and Brimonidine Tartarate obtained by MODDE software from 19 DOE experimental run on SiliaBond C18 (50x 2.1 mm, 1.8µm)



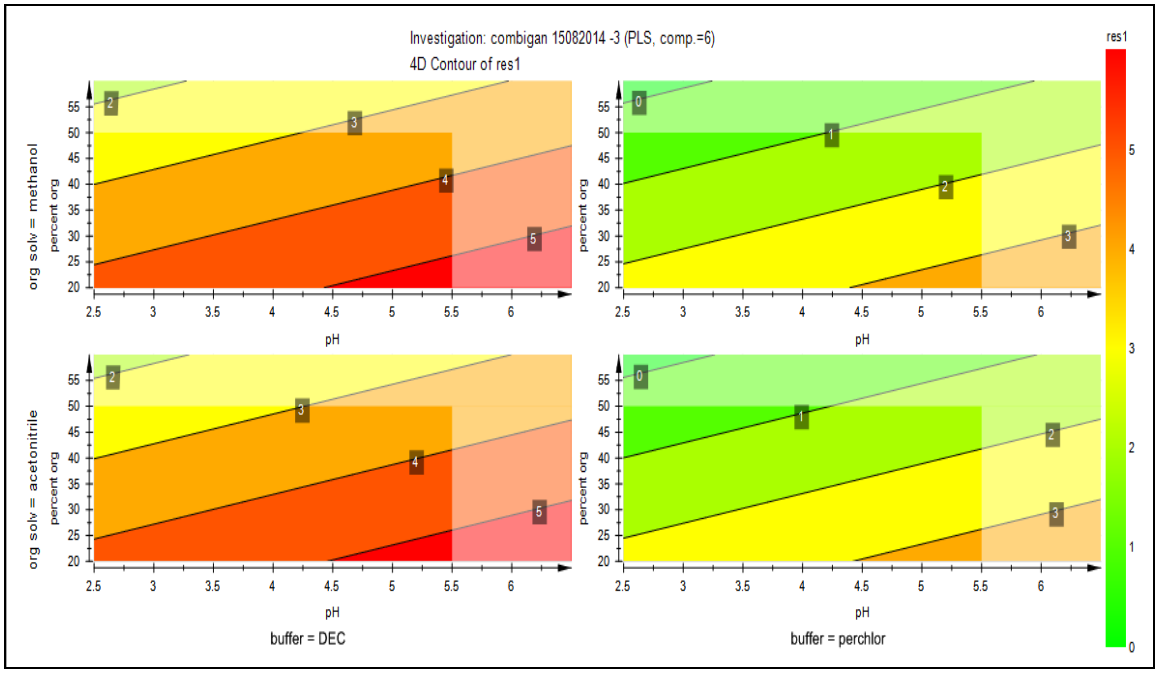
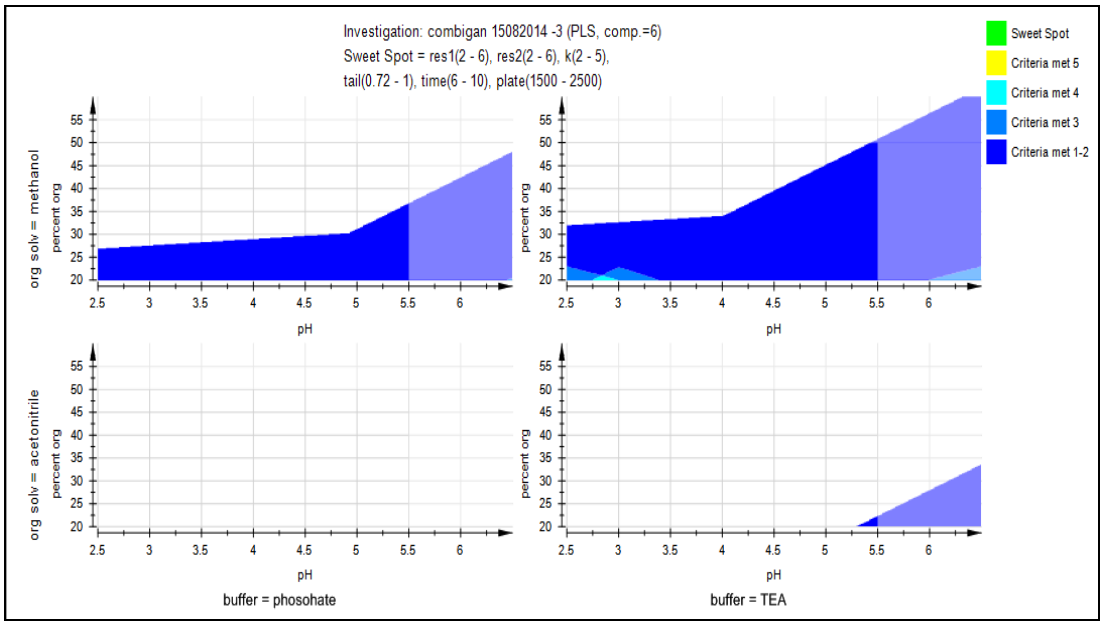


Fig 3c-d: 4D Contour plot (Resolution Map) for resolution between Maleic acid and Brimonidine Tartarate obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x4.6 mm, 2.6µm)



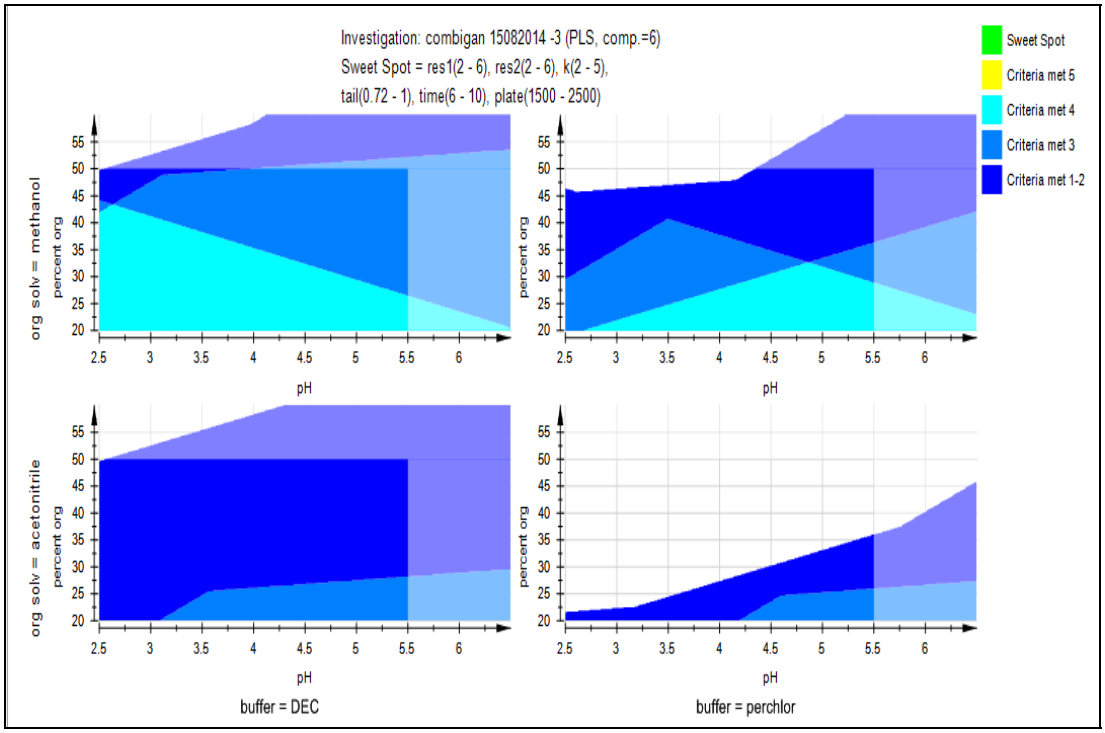
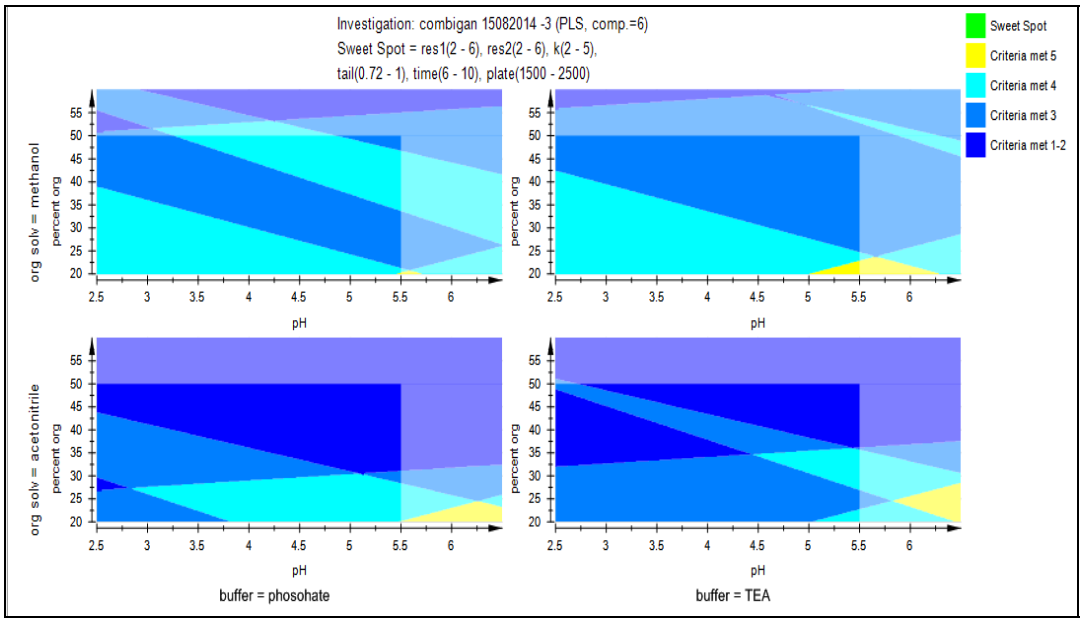


Fig 4a-b: 4D sweet spot plot (Design Space) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x4.6 mm, 2.6µm)



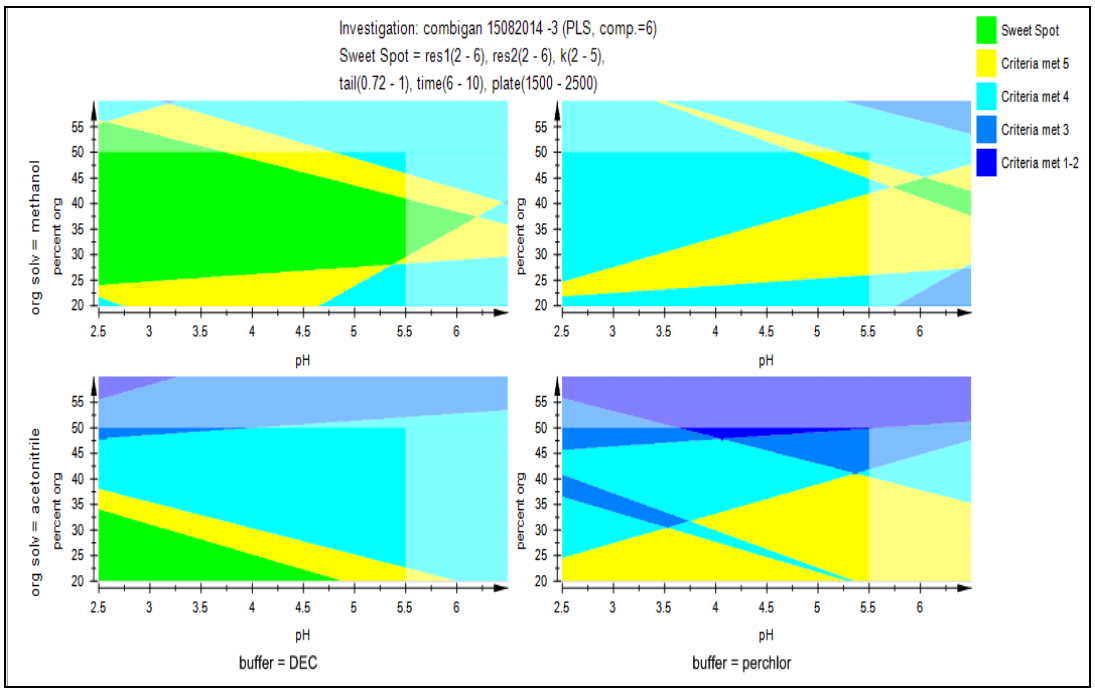


Fig 4c-d: 4D sweet spot plot (Design Space) obtained by MODDE software from 19 DOE experimental run on Phenomenex Kinetex C18 (50x4.6 mm, 2.6µm)

These reasons led to select high concentration of benzalkonium chloride from 1- 10 μgmL^{-1} to be detected and quantified as well as high concentration of Brimonidine and Timolol (10 - 100 and 25-225 μgmL^{-1}) respectively with 10 µL injection volume.

As a result of high absorptivity of Brimonidine and Timolol at 210nm, a problem in linearity and accuracy was appeared as shown in Fig 5.

Wavelength was changed to 254 or 300 nm but it did not overcome the problem. So, wavelength 320nm was tried and linearity and precision problems were resolved as shown in Fig 6A.

Optimized method was verified by method validation especially method robustness as mentioned above according to ICH guideline Q2 (R1) [17].

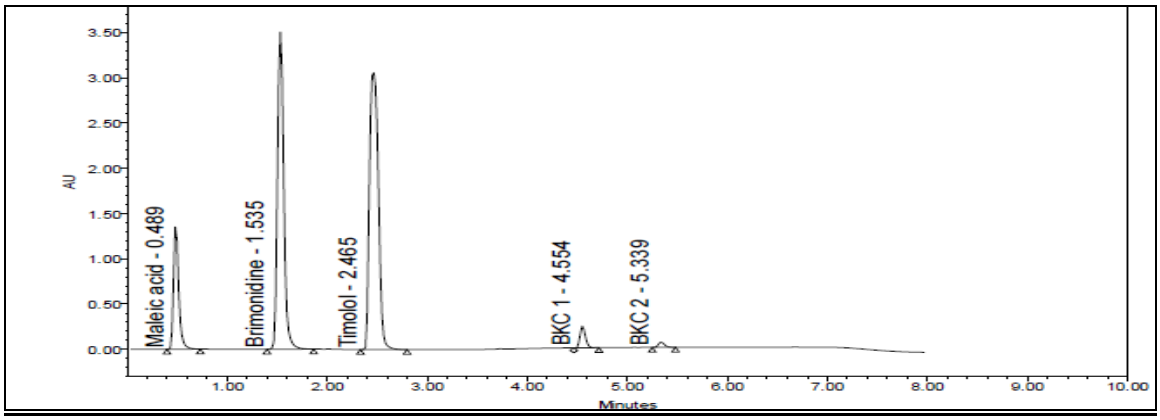
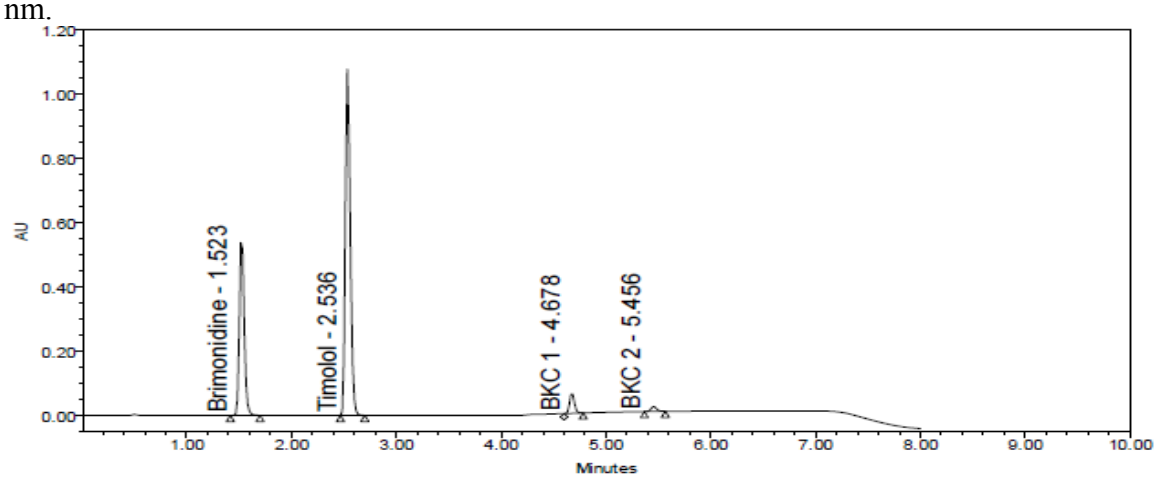
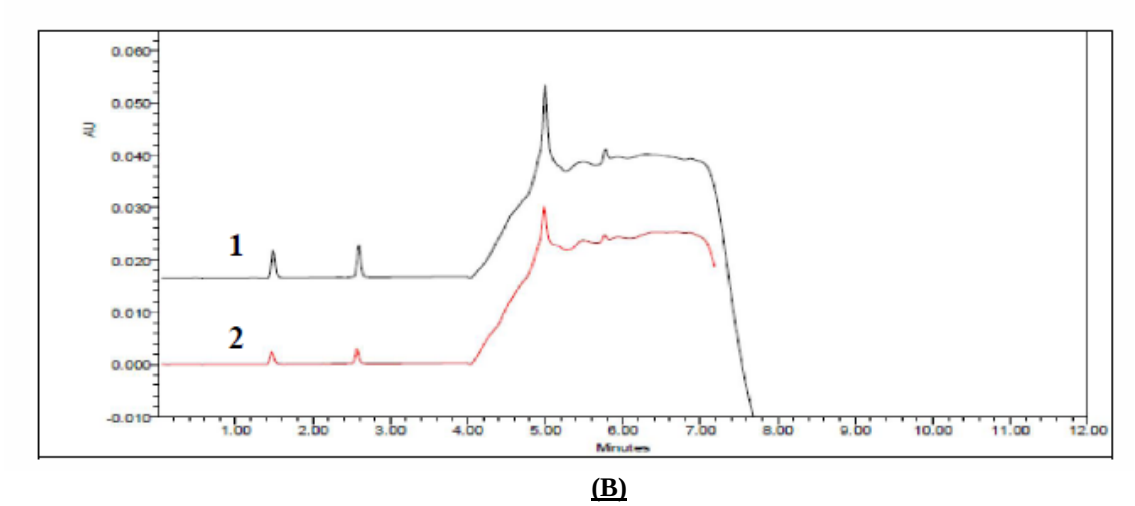


Fig 5: Typical UPLC chromatograms obtained from 20 µL injections of Brimonidine Tartrate (1.52 min.), Timolol Maleate (2.53 min.) and Benzalkonium Chloride (4.55 and 5.33 min.) respectively under optimized chromatographic conditions except wavelength was set at 210 nm.



(A)



(B)

Fig 6A,B: Typical UPLC chromatograms obtained from 20 µL injections of Brimonidine Tartrate (1.52 min.), Timolol Maleate (2.53 min.) and Benzalkonium Chloride (4.68 and 5.46 min.) respectively under optimized chromatographic conditions. (A) At 100% conc. Level of all compounds and tablet. (B) At DL and QL

3. Results and discussion

3.1. Method validation

3.1.1. Specificity

A Bulk of Combigan® ED (solution contains excipients only) without Benzalkonium Chloride was prepared by mixing its excipients like sodium phosphate, monobasic; sodium phosphate, dibasic and purified water then the bulk was injected under previous condition. Representative chromatogram showed that the bulk has negligible contribution after the void volume at the method detection wavelengths i.e. it did not interfere with developed method as shown in Fig 6A.

The method was also evaluated by assessing degradation products which obtained from stress studies involving acid, base, peroxide, and heat stored under ICH stability conditions. Degradation products did not interfered with the analysis of Brimonidine Tartrate, Timolol and Benzalkonium Chloride as shown later [17].

3.1.2. Linearity and range

Ten Concentrations were chosen in the ranges (1-100 and 2.25-225 μgmL^{-1}) for corresponding levels of (2-200%) w/w of the nominal analytical concentration of Brimonidine Tartrate and Timolol respectively. Ten Concentrations were chosen in the ranges (1- 10 μgmL^{-1}) for corresponding levels of 20 -200% w/w of the nominal analytical concentration of Benzalkonium chloride.

The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were {Y = 43.40 X +8.799 (r= 0.9997), Y = 33.62 X+13.09 (r= 0.9998)} and Y = 61.93 X-3.8523 r= 0.9995)} for Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively. Where Y is the peak area of standard solution and X is the drug concentration as shown in Table 4 [17].

Table 4: Calibration data was resulted from method validation of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively

Item	Brimonidine Tartrate	Timolol Maleate	Benzalkonium Chloride
Linear range (μgmL^{-1})	1-100	2.25-225	1-10
Detection limit (μgmL^{-1})	0.1	0.17	0.28
Quantitation limit (μgmL^{-1})	0.3	0.51	0.85
Regression data			
No.	10	10	10
slope (b)	43401	33623	61933
Standard deviation of the slope	6.51	5.29	5.77
intercept (a)	8799	13092	-3852
Standard deviation of the intercept	354.6	1712.4	528.6
correlation coefficient ®	0.9997	0.9998	0.9995
Standard error of regression	26.79	36.7	5.118

(Y = a + bC, where C is the concentration of the compound (µgmL-1) and Y is the drug peak area)

3.1.3. Precision

The precision of the assay was investigated by measurement of both repeatability and intermediate precision.

3.1.3.1. Repeatability

Repeatability was investigated by injecting a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each) and percentage SD was calculated in Table 5 [17].

3.1.3.2. Intermediate precision

In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at specified range for the procedure (e.g., 3 concentrations/3 replicates each) of the test concentration and percentage SD was calculated in Table 5 [17].

Table 5: Repeatability and Intermediate precision and Accuracy (Recovery %) of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride

Drug Name	Conc.	Brimonidine Tartrate		Timolol Maleate		Benzalkonium Chloride	
		AV±SD μgmL^{-1}	AV±SD %	AV±SD μgmL^{-1}	AV±SD %	AV±SD μgmL^{-1}	AV±SD %
Repeat-ability	75%	29.78 ±0.08	99.25±0.28%	74.86 ±1.10	99.82±0.10%	3.02 ±0.01	100.62±0.07%
	100 %	40.52 ±0.08	101.3±0.09%	100.91 ±3.30	100.91±0.30%	4.05 ±0.08	101.25±2.05%
	125%	50.60 ±0.13	101.2±0.26%	124.14 ±2.60	99.32±0.20%	5.08 ±0.05	101.67±1.03%
Intermediate precision	75%	29.91 ±0.18	99.69±0.14%	74.89 ±0.29%	99.85±0.39%	3.03 ±0.02	100.99±0.53%
	100 %	40.52 ±0.06	101.30±0.70%	100.37 ±0.64%	100.37±0.64%	4.05 ±0.05	101.19±1.30%
	125%	50.34 ±0.35	100.68±0.98%	123.35 ±0.90%	98.68±0.72%	5.03 ±0.08	100.61±1.63%
Accuracy	75%	30.35±0.07	101.19±0.23%	76.42 ±0.42	101.90±0.60%	2.97 ±0.02	98.85±0.51%
	100 %	40.73±0.04	101.83±0.09%	101.57 ±0.14	101.57±0.10%	4.08 ±0.03	101.98±0.82%
	125%	50.30±0.21	100.60±0.42%	124.68 ±0.35	99.75±0.30%	4.91 ±0.03	98.13±0.67%

N.B. (75%, 100% and 125%) Concentration of Brimonidine Tartrate, Timolol and Benzalkonium Chloride are {(30, 40, 50) and (75, 100, 125), (3, 4 and 5) μgmL^{-1}} respectively.

4.1.4. Accuracy

In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at specified range for the procedure (e.g., 3 concentrations/3 replicates each) of the test concentration and percentage SD was calculated in Table 5 [17].

4.1.5. Limits of detection and Limits of quantitation

According to the ICH recommendations, determination of limits of detection and quantitation was based on the standard deviation of the y-intercepts of regression lines (n=3) and the slope of the calibration plots [17] as shown in Table 4 and Fig 6B.

4.1.6. System suitability tests

System suitability tests were used to verify that the resolution and reproducibility were adequate for the performed analysis. The system suitability tests included number of theoretical plates, resolution, peak tailing, capacity factor and selectivity factor. Results are revealed in Table 6.

Table 6: System suitability parameters of all drugs were obtained from Method Validation

Drugs/Parameters	Theoretical Plates (N)	Resolution (R)	Capacity Factor (K)	Tailing Factor (T)	Selectivity (α)
Brimonidine	5435.06	-	0.52	1.18	-
Timolol	12615.59	11.34	1.54	1.14	2.94
Bkc1	54594.71	24.54	3.68	1.02	2.39
Bkc2	49316.54	8.33	4.46	1.14	1.21

4.1.7. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage [17]. Robustness was tested by studying the effect of changing mobile phase pH by ± 0.2, the percentage of organic solvent (methanol) in the mobile phase by ± 2 %, temperature ± 2 C°, wavelengths ± 2 nm and flow rate ± 0.05 $mLmin^{-1}$ had no significant effect on the chromatographic resolution of the method as shown in Table 7 and Fig 7.

Table 7: Effect of Changes of Some Parameters on Resolution during Method Robustness

Parameters	Flow Rate		PH		Methanol%		Wavelengths		Temperature	
Value	0.55	0.65	2.8	3.2	48	52	318-208	322-212	48	52
Brimonidine	-	-	-	-	-	-	-	-	-	-
Timolol	10.40	10.13	9.86	10.59	10.59	9.86	10.51	10.57	9.44	9.47
Bkc1	20.71	21.87	21.98	21.47	21.47	21.98	21.42	22.19	20.84	21.59
Bkc2	8.30	7.30	6.42	9.52	9.52	6.42	7.71	7.94	9.03	8.31

4.1.8. Stability of analytical solution

As a part of evaluation of robustness, solution stability was evaluated by monitoring the peak area response. Standard stock solutions in water were analyzed right after its preparation 1, 2 and 3 days after at room temperature. The change in standard solution peak area response over 3 days was (0.59, 0.63 and 1.30 %) for Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively. Their solutions were found to be stable for 3 days at room temperature at least.

4.2. Application on pharmaceutical Preparation

The proposed methods were successfully used to determine Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively in Combigan® ED. Four replicate determinations were performed. Satisfactory results were obtained for each compound in good agreement with label claims. The results obtained were compared statistically with those from published methods [6, 18] by using Student's t-test and the variance ratio F-test. The results showed that the t and F values were smaller than the critical values. So, there were no

significant differences between the results obtained from this method and published methods as shown in Table 8.

Table 8: Statistical comparison of the proposed and published methods for determination of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively in their dosage forms by reported method (T- student test) and (F –test for variance)

Drug name	Recovery ± SD Proposed methods	Reference method	Calculated t- values	Calculated F- values
Brimonidine Tartrate	101.85±1.14	100.23±2.15	1.68	0.28
Timolol Maleate	102.64±1.19	101.26±2.45	1.15	0.24
Benzalkonium Chloride	98.77±1.70	97.95±3.03	1.79	0.31

(Where the Tabulated t-values and F -ratios at p = 0.05 are 2.776 and 6.39)

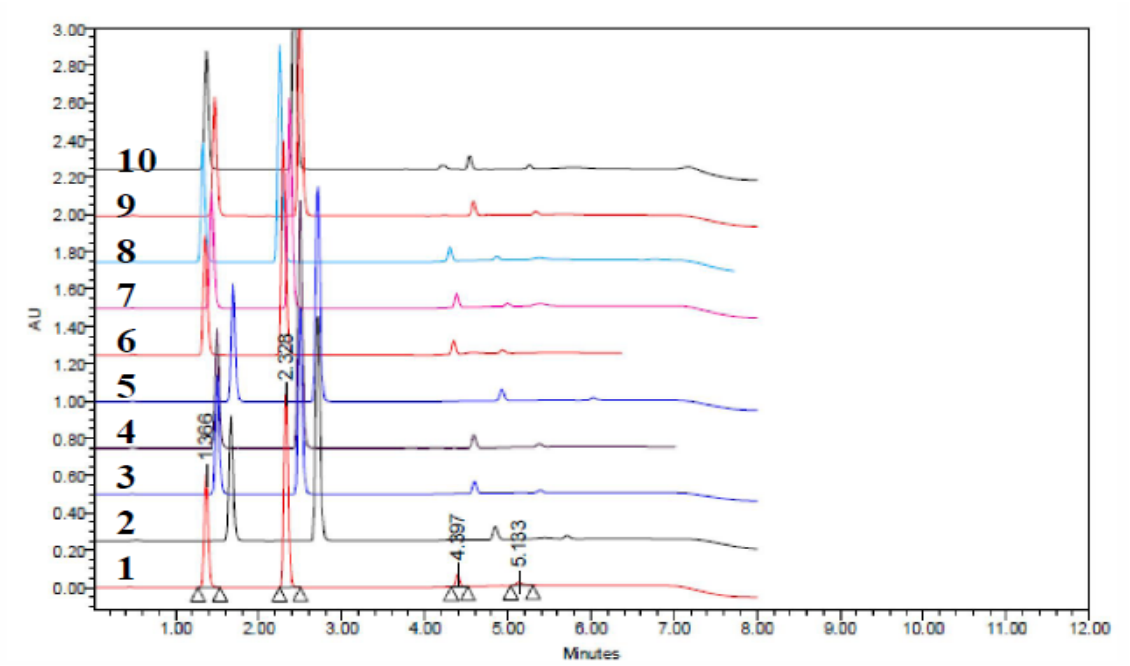


Fig 7: Typical UPLC chromatograms obtained from 20 µL injections of Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride respectively under optimized chromatographic conditions. (Ascending order)
1, 2- at 0.55 and 0.65 mL /min
3, 4- at 48, 52 C°
5, 6- At 318 and 322 nm
7, 8- at 48% and 52% of methanol
9, 10- at buffer pH = 2.8 and 3.2

4.3. Forced degradation studies

Brimonidine Tartrate, Timolol Maleate and Combigan® ED were subjected to various stress condition.

In acidic and alkaline conditions, Brimonidine Tartrate and Timolol Maleate about 20 and 40 % of degradation respectively as shown in Table 9, Fig 8 and 9.

In oxidative conditions, both drugs were completely degraded under 3 % H_2O_2 at 75C° after 6 hours as shown in Table 9 and Fig 10.

Table 9: Results of degradation percent of Brimonidine Tartrate and Timolol Maleate obtained from stress test condition

Stress test condition	Brimonidine Tartrate		Timolol Maleate	
	Peak Area	Assay after Degradation %	Peak Area	Assay after Degradation %
Std	725920	100	1114217	100
HCl (1M) at 75 ºC / 6 hours	574382	79.12	711935	63.89
NaOH (1M) at 75 ºC / 6 hours	561503	77.35	648614	58.21
H_2O_2 (3%) at 75 ºC / 6 hours	0	0	0	0
Heat at 75 ºC at 75 ºC / 6 hours	570338	78.56	1102052	98.90
Light	580576	79.97	981300	88.07

Brimonidine was degraded in 3 % H_2O_2 after 5 minutes at room temperature but Timolol was less labile to it at room temperature.

Timolol was more stable than brimonidine (20% degradation) under neutral degradation (Thermal degradation) as shown in Table 9 and Fig 11.

Both Brimonidine Tartrate and Timolol Maleate were affected by photolytic degradation about 20% and 12% respectively as shown in Table 9 and Fig 12.

From previous experiments, Brimonidine was labile for degradation than Timolol.

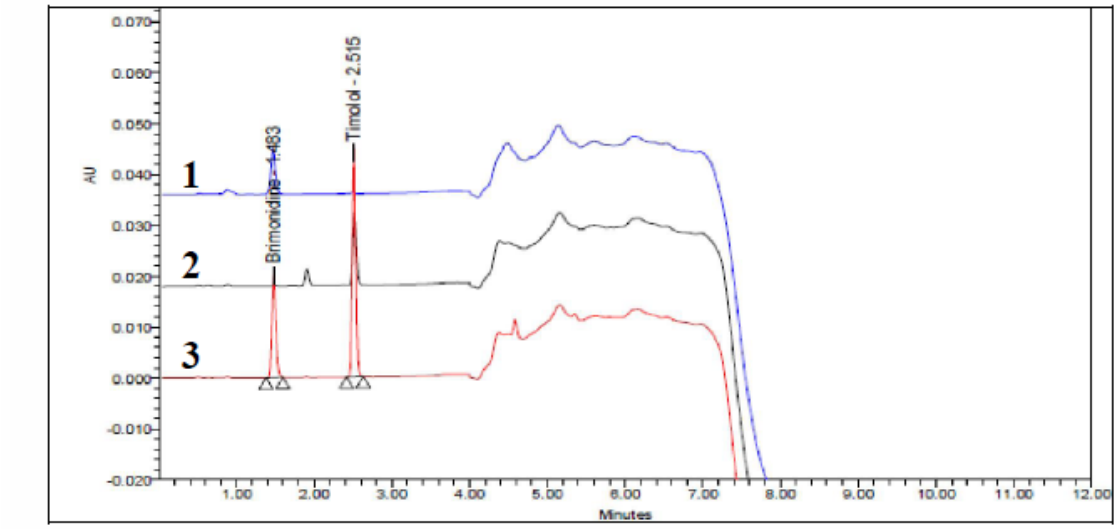


Fig 8: Typical UPLC chromatograms obtained from 10 µL injections of solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED which were subjected to acidic condition under optimized chromatographic conditions. (Descending order)

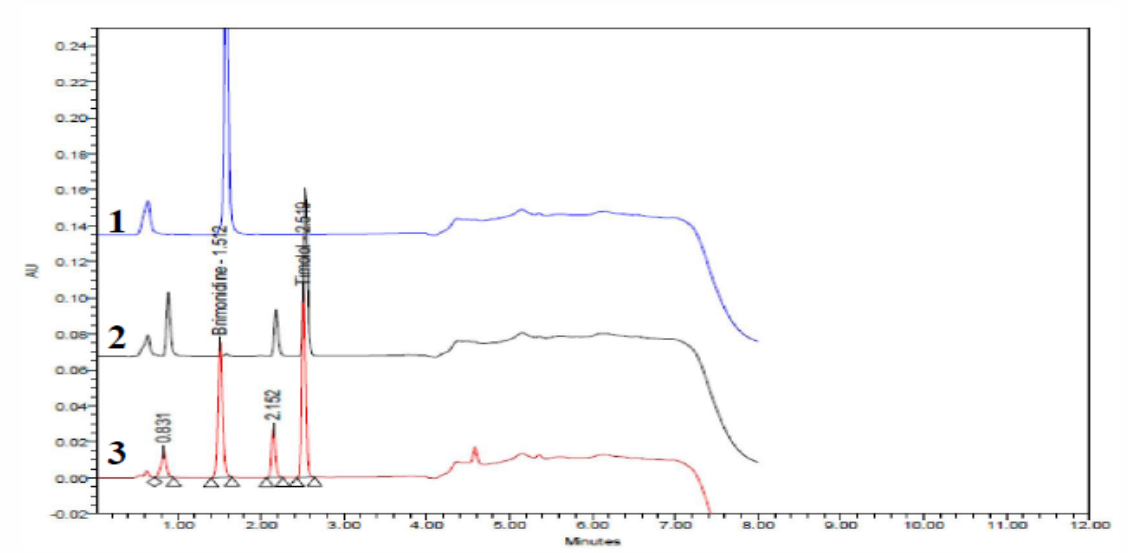


Fig 9: Typical UPLC chromatograms obtained from 10 µL injections of solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED which were subjected to alkaline condition under optimized chromatographic conditions. (Descending order)

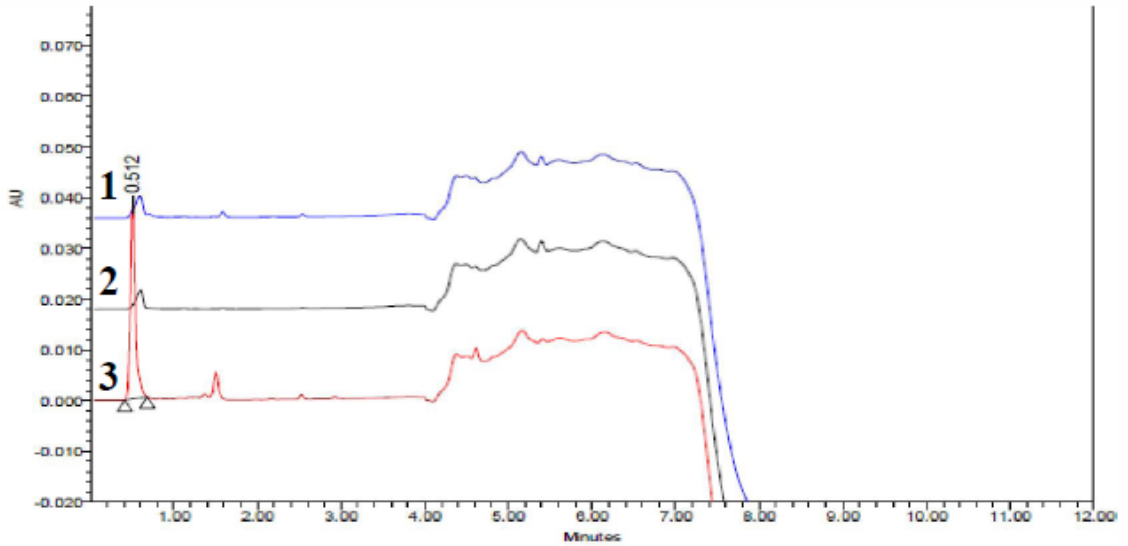


Fig 10: Typical UPLC chromatograms obtained from 10 µL injections of solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED which were subjected to oxidative condition under optimized chromatographic conditions. (Descending order)

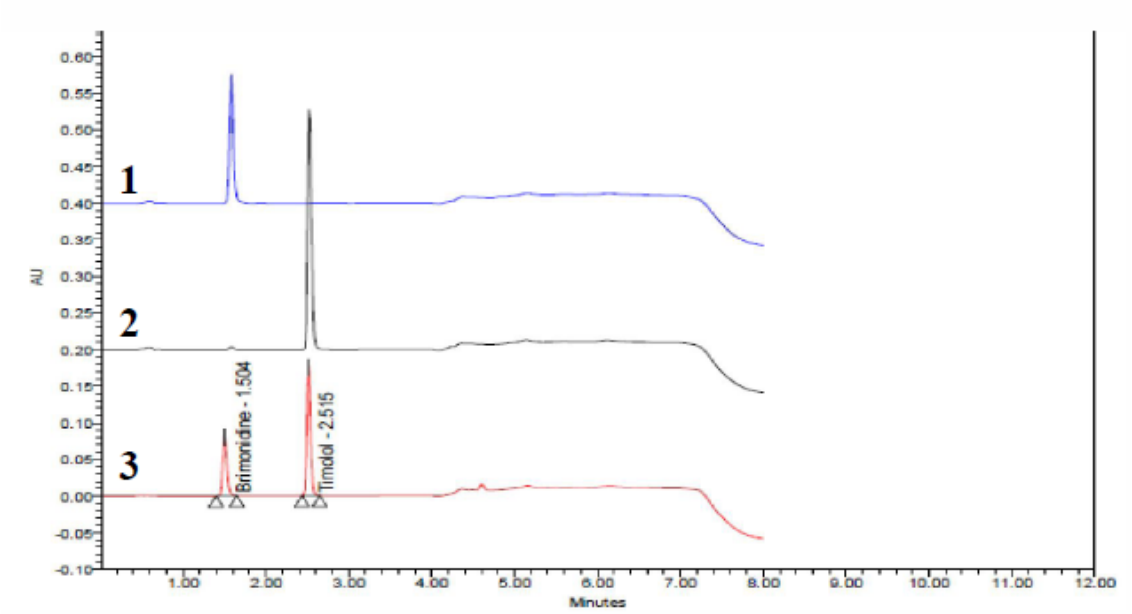


Fig 11: Typical UPLC chromatograms obtained from 10 µL injections of solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED which were subjected to thermal condition under optimized chromatographic conditions. (Descending order)

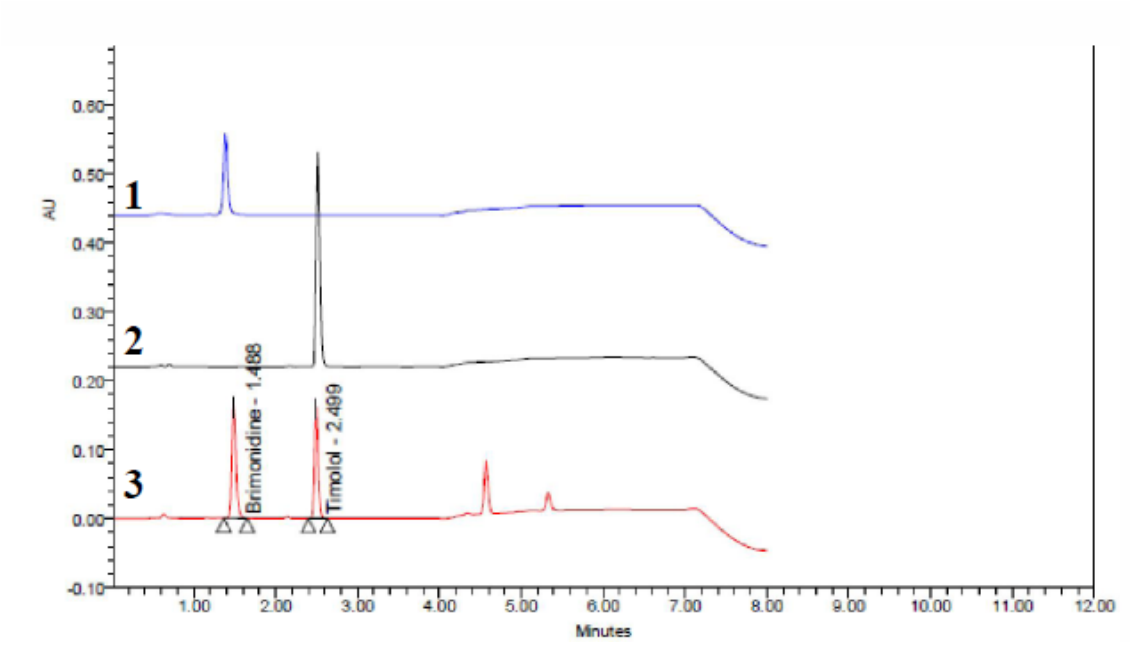


Fig 12: Typical UPLC chromatograms obtained from 10 µL injections of solutions of Brimonidine Tartrate, Timolol Maleate and Combigan® ED which were subjected to photolytic degradation under optimized chromatographic conditions. (Descending order)

4. Discussion

New developed method has several merits than other published methods in literature; it utilized DOE technique in development and optimization which led to high robust method.

It determines Brimonidine Tartrate and Timolol Maleate as well as Benzalkonium Chloride in presence of its degradation products in a single run and it can be used as a stability indicating method. Good peaks shape and resolution between studied drugs.

It was performed by UPLC technique which led to shorter retention time (6 minutes). Specificity was proven clearly after degradation products of both drugs had been separated.

5. Conclusion

A simple, accurate, precise, robust and reliable stability indicating UPLC method has been established for simultaneous determination for Brimonidine Tartrate, Timolol Maleate and Benzalkonium Chloride in ophthalmic drops

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