Original Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BRIMONIDINE TARTRATE AND TIMOLOL MALEATE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Brimonidine Tartrate and Timolol Maleate are used in treatment of glaucoma by decreasing intra ocular pressure. A Validated HPLC method was developed for the assay of them. The method was performed on BDS HYPERSIL Cyano column (250x4.6 mm, 5µ) and the mobile phase consisted of Ammonium acetate (pH 5.0, 0.01M) - Methanol (40:60, V/V) which pumped at a flow rate equals to 1.5 ml/min at ambient temperature. 20 µl of drugs sample solutions were monitored at two fixed wavelengths (lambda = 254.0 nm for Brimonidine Tartrate and 300.0 nm for Timolol Maleate). 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; HPLC.

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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. Glaucoma 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 available 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]

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 (Fig.1), it is a non-cardioselective beta blocker. It is reported to lack intrinsic sympathomimetic and membrane-stabilizing activity. Timolol Maleate is used in the management of glaucoma, hypertension, angina pectoris and myocardial infarction [1]. Brimonidine is 5-Bromo-N-(4,5-dihydro-1H-imidazol-2-yl) quinoxalin-6-amine(Fig.1), it is a selective alpha -adrenoceptor agonist, is licensed for the reduction of intra-ocular pressure in open-angle glaucoma or ocular hypertension in patients for whom beta-blockers are inappropriate; it may also be used as adjunctive therapy when intra-ocular pressure is inadequately controlled by other anti-glaucoma therapy[1].

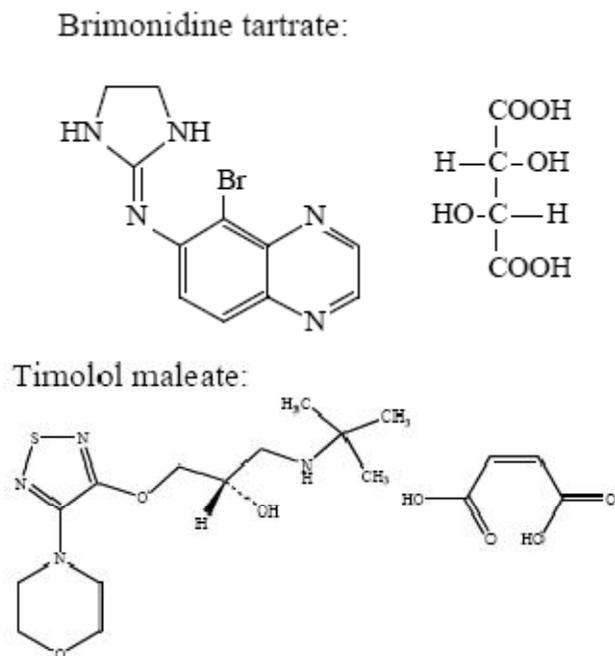Brimonidine tartrate:

Timolol maleate:

Fig.1. Structures of Brimonidine Tartrate and Timolol Maleate respectively

Eye drops (Combigan eye drop) containing Timolol Maleate equivalent to 0.5% of Timolol and 0.2% of Brimonidine are instilled twice daily to reduce raised intra-ocular pressure in open-angle glaucoma and ocular hypertension.

Literature review reveals that different methods have been reported for estimation of both drugs individually like, RP-HPLC for the Analysis of Brimonidine in Ophthalmic Formulations [2,3], in blood serum and aqueous humor of the eye [4] and LC/MS/MS HPLC for the Analysis of Brimonidine in Ocular Fluids and Tissues.[5] Three papers discussed stability of Brimonidine tartrate.[6-8] other analytical techniques were reported for determination of Brimonidine tartrate like HPTLC[9],GC/MS[10] and CE[11].

Timolol Maleate is listed in USP which described RP-HPLC for determination Timolol maleate in eye drops [12]. BP estimated Timolol Maleate potentiometrically [13].

Literature review reveals several methods have been reported for the estimation of Timolol Maleate in biological fluids [14-15] and there are some methods reported by voltametry [16], spectroscopy [17], HPTLC [18] HPLC [19-20], UPLC [21] and capillary electrophoresis [22]. Two methods were only reported for estimation of this combination, the first is HPTLC [23] and the other is HPLC method [24]. The latter does not fulfill all requirements of validation which will be discussed later. Development of HPLC method for simultaneous estimation of this combination is a must and our scope that our new method will fulfill all requirements of validation according to ICH guidelines. It should be characterized by simplicity, fast analysis, and sensitivity and suitable for routine pharmaceutical analysis.

2. EXPERIMENTAL

2.1. Instrumentation

Analysis was performed on a chromatographic system of WATERS 2695 separation module connected to WATERS 2487 UV/VIS detector. The system equipped by Empower PC program. The chromatographic separation was achieved on BDS HYPERSIL Cyano column (250x4.6 mm, 5µ).

2.2. Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. Ammonium acetate and glacial acetic acid were supplied by (Merck, Darmstadt, Germany), Methanol HPLC grade was supplied by (Fischer scientific, U.K.) and Distilled water.

(Note: The water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA).

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

2.3. Pharmaceutical preparation

Combigan eye drops, Allergan (U.S.A) contains (Brimonidine Tartrate 0.2% and Timolol (as Maleate) 0.5%) B.NO: E64005.

2.4. Chromatographic condition

20 µl of drugs sample solutions were monitored at two fixed wavelengths (lambda = 254.0 nm for Brimonidine Tartrate and 300.0 nm for Timolol Maleate). Liquid chromatography was performed on BDS HYPERSIL Cyano column (250x4.6 mm, 5µ) and the mobile phase consisted of Ammonium acetate (pH 5.0, 0.01M) - Methanol (40:60, V/V) which pumped at a flow rate equals to 1.5 ml/min at ambient temperature.

Ammonium acetate (0.01 M) was prepared by dissolving 0.77 g Ammonium acetate in approximately 950 ml distilled water. The pH was adjusted to 5.0 with glacial acetic acid. Water was added to 1000 ml. Mobile phase was filtered through a 0.45 µl Nylon membrane filter (Millipore, Milford, MA, USA) under vacuum and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage.

2.5. Preparation of stock standard solutions

Stock standard solutions containing 0.2, 0.68 mg/ml of   Brimonidine Tartrate and Timolol Maleate (equivalent to 0.5 mg/ml of Timolol) respectively were prepared by dissolving 20, 68 mg of each in distilled water in 100 ml volumetric flask respectively. It was then sonicated for 5 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 solutions in the concentration ranges (4-24 and 10-60 µg/ml) for of   Brimonidine Tartrate and Timolol respectively. Each solution (n=5) 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

Take 1 ml of Combigan E/D into 100 ml V.F. then complete with distilled water.

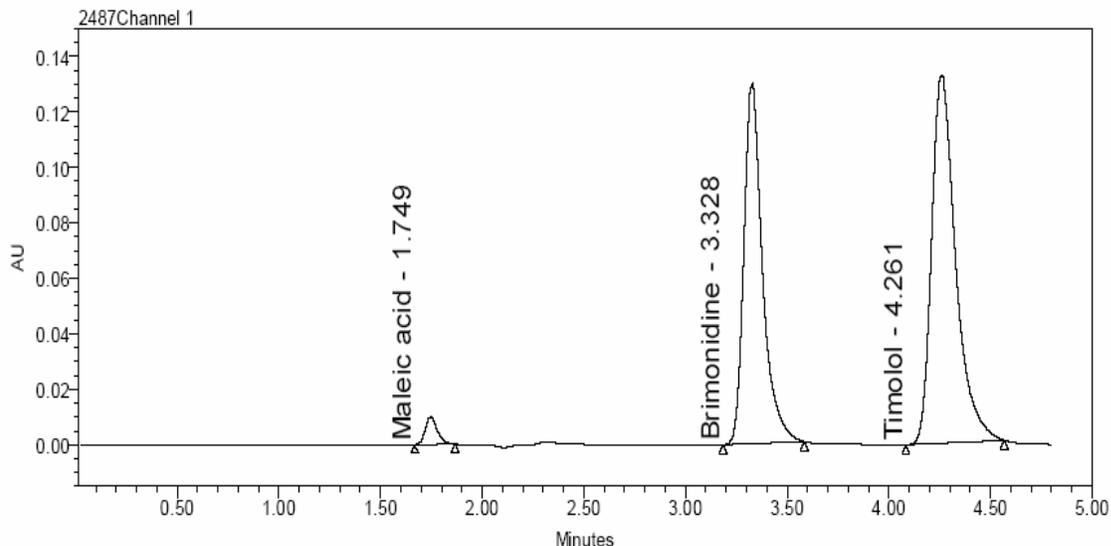Test solutions were analyzed under optimized chromatographic conditions and chromatogram is depicted in (Fig.2).

Fig.2. Typical HPLC chromatograms obtained from 20 µl injections of Brimonidine Tartrate (3.86 min.) and Timolol Maleate (3.86 min.) respectively under optimized chromatographic conditions.

3. METHOD VALIDATION

3.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc [25]. A Bulk of Combigan E/D (solution contains excipients only) was prepared by mixing its excipients like benzalkonium chloride 0.005%; 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.

3.2. Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. For the establishment of linearity, a minimum of 5 concentrations is recommended [25]. Five Concentrations were chosen in the ranges (4-24 and 10-60 µg/ml) for corresponding levels of 20-120% w/w of the nominal analytical concentration of Brimonidine Tartrate and Timolol respectively. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were {Y = 42167 X - 4937 (r= 0.9999) and Y = 22395X - 8914 (r= 0.9998)} for Brimonidine Tartrate and Timolol Maleate respectively. Where **Y** is the peak area of standard solution and **X** is the drug concentration (Fig.3).
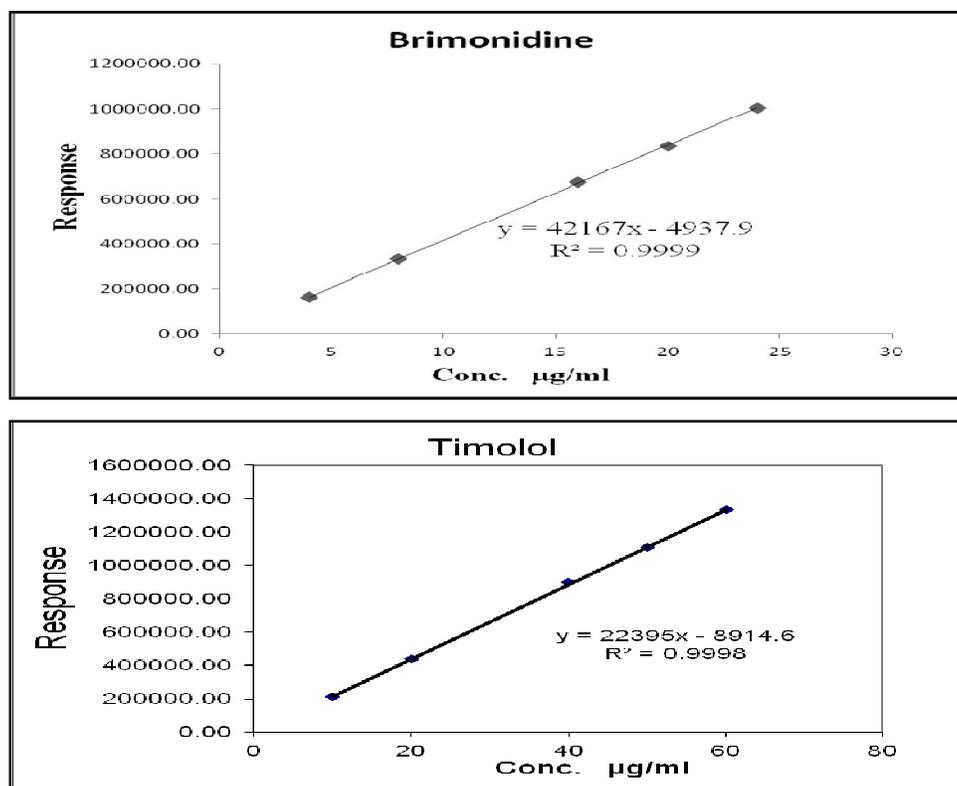
Fig.3. Calibration plot of Brimonidine Tartrate and Timolol Maleate

3.3. Precision

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

3.3.1. Repeatability

Repeatability was investigated by injecting a minimum of 6 determinations at 100% of the test concentration and percentage SD were calculated in Table 1.

3.3.2. Intermediate precision

In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at 100% of the test concentration and percentage SD were calculated Table 1.

3.4. Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (80,100 and 120%). Accuracy was reported as percent recovery by the assay of known added amount of analyte in the sample (Table 1).

Table 1: Repeatability and Intermediate precision and Accuracy (*Recovery %)* of Brimonidine Tartrate and Timolol Maleate respectively

Drug name		Brimonidine Tartrate		Timolol Maleate	
		AV±SD mg/ml	AV±SD %	AV±SD mg/ml	AV±SD %
Repeatability		19.98±0.19	99.88±0.94%	49.90±0.50	99.81±1.00%
Intermediate precision		19.99±0.32	99.9±1.59%	49.78±0.84	99.56±1.67%
Accuracy	*80%*	*15.99±0.13*	*99.94±0.78%*	*40.21±0.43*	*100.53±1.06%*
& Recovery	100%	20.06±0.15	100.32±0.75%	50.01±0.51	100.01±1.02%
%	120%	24.20±0.19	100.83±0.80%	60.34±0.43	100.57±0.72%

3.5. 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 [25] (Table 2).

Table 2: Calibration data was resulted from method validation of Brimonidine Tartrate and Timolol Maleate respectively

Item	Brimonidine Tartrate	Timolol Maleate
Linear range (µg/ml)	4-24	10-60
Detection limit (µg/ml)	0.05	0.09
Quantitation limit (µg/.ml)	0.15	0.29
Regression data		
No.	5	5
slope (b)	42167	22395
Standard deviation of the slope	56.36	36.67
intercept (a)	4937	8914
Standard deviation of the intercept	637.86	641.36
correlation coefficient ®	0.9999	0.9998
Standard error of regression	0.09	0.3

(Y = a + bC, where C is the concentration of the compound (µg/ml) and Y is the drug peak area)

3.6. 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 [25]. Robustness was tested by studying the effect of changing mobile phase pH by ± 0.5, the percentage of organic solvent (methanol) in the mobile phase by ± 5 %, temperature ± 5 C, wavelengths ± 5 nm and flow rate ± 0.1 ml/min had no significant effect on the chromatographic resolution of the method.

3.6.1. Stability of analytical solution

Also as part of evaluation of robustness, solution stability was evaluated by monitoring the peak area response. Standard stock solutions in methanol 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 (1.01 and 0.89 %) for Brimonidine Tartrate and Timolol Maleate respectively. Their solutions were found to be stable for 3 days at room temperature at least.

4.0. Application on pharmaceutical Preparation

The proposed methods were successfully used to determine Brimonidine Tartrate and Timolol Maleate respectively in Combigan E/D. Five 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 method [24] 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 (Table 3).

Table 3: Statistical comparison of the proposed and published methods for determination of Brimonidine Tartrate and Timolol Maleate 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	101.41±0.95	100.85±1.00	2.49	0.94
Timolol Maleate	101.40±1.32	101.23±1.16	1.13	1.28

(Where the Tabulated t-values and F -ratios at $p = 0.05$ are 2.57 and 5.05)

5. RESULTS AND DISCUSSION

5.1. Optimization of chromatographic condition

Several trials were carried out to obtain optimized chromatographic condition for simultaneous determination of Brimonidine Tartrate and Timolol Maleate in their pharmaceutical preparations. Firstly, maximum absorption wavelengths (254,300 nm) for Brimonidine Tartrate and Timolol Maleate were selected by scanning from 350-200 nm under UV (Fig.4).

Ammonium acetate buffer has no effect on absorption at wavelength more than 250 nm [26]. Low conc. of buffer (0.01M) is adequate for most reversed phase applications. This concentration is also low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase [27] .PH of acetate buffer was examined, at pH= 3 interference between maleic acid peak and solvent peak had occurred but interference disappeared at pH=5. Different percentages of organic solvent (methanol) were tried with acetate buffer to reach optimum separation with good resolution.60% methanol in mobile phase gave adequate separation and resolution. Using Cyano column enabled us to produce sharp and symmetric peaks because it is better in separation of drug containing basic functionality like amino compounds [28].
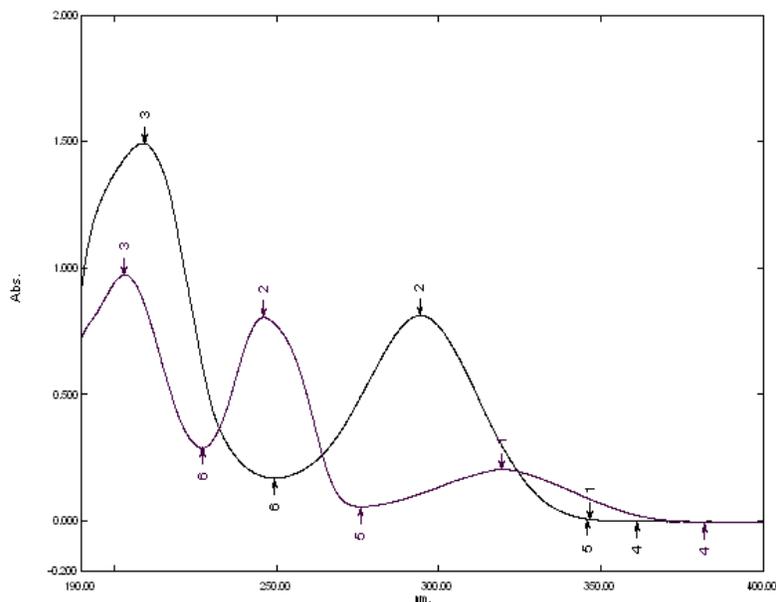
Fig.4. Typical UV spectrum of Brimonidine Tartrate and Timolol Maleate

6. CONCLUSION

A simple, accurate, precise, robust and reliable LC method has been established for simultaneous determination for Brimonidine Tartrate and Timolol Maleate respectively in bulk and in their pharmaceutical dosage form. The method has several advantages more than Arun Phagot [24]

1- We selected maximum absorbance wavelengths (254,300 nm) for Brimonidine Tartrate and Timolol Maleate respectively but he selected 295 nm for both drugs.

2- More sensitive method has LOD range (0.02-0.05) µg/ml and LOQ range (0.06-0.15) µg/ml for Brimonidine Tartrate and Timolol Maleate respectively.

3- Method validation has been achieved within ICH limits unlike Arun Phagot [24], there are some deviations like some recovery percentages are less than 98%, correlation coefficients are less than 0.999 and trials in robustness shows it is not robust but it is critical method due to narrow limits as in pH and his information about change in wavelengths which leads to decease peak sharpness is not correct.

4- Our method clearly separated maleic acid from other compounds.

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