Development and Validation of TLC-densitometry Method for Simultaneous Estimation of Brimonidine tartrate and Timolol maleate in Bulk and Pharmaceutical Dosage Form

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Abstract
A new simple, precise, accurate and selective TLC-densitometry method has been developed for simultaneous determination of brimonidine tartrate and timolol maleate in pharmaceutical formulation. Chromatographic separation was performed on aluminum plate precoated with silica gel RP-18 F254 using methanol: water: triethylamine (2.0:2.0:0.2 v/v) as mobile phase. Detection was carried out densitometrically at 264 nm. The Rf value of brimonidine tartrate and timolol maleate were 0.23 and 0.63, respectively. The reliability of the method was assessed by evaluation of linearity which was found to be 300 – 1800 ng/spot for brimonidine tartrate and 1000 - 6000 ng/spot for timolol maleate. Accuracy of the method was accessed by percentage recovery and found to be 99.77 ± 0.71 % for brimonidine tartrate and 99.87 ± 0.86 % for timolol maleate. The method can be used for routine analysis of brimonidine tartrate and timolol maleate in pharmaceutical formulation.

Keywords: Brimonidine tartrate; Timolol maleate; TLC/densitometry; Validation.

Introduction
Brimonidine tartrate chemically, 5-bromo-6-(2-imidazoline-2-yl amino) quinoxaline L-tartrate is a α2-adrenoreceptor agonist, derived from clonidine, as aproclonidine but less lipophilic than clonidine, is used to treat open angle or ocular hypertension. It leads to a decreased production of aqueous humor and increases the amount that drains from the eye. Timolol maleate is a nonspecific β-adrenergic blocker. It was the first β-blocker to be used as an antiglaucoma agent. Both the drugs are official in Merck Index and Martindale [1,2]. The chemical structures are shown in (Figure 1).

Reverse phase liquid chromatography method has been reported for the quantification of brimonidine tartrate in eye drops [3]. Two LC/MS/MS methods are also reported for quantification of brimonidine tartrate in biological samples and in eye drops [4,5]. Its synthesis and detection by mass spectrophotometry method is also reported [6]. A capillary electrophoretic analysis of brimonidine tartrate in biological fluid is also reported [7]. A stability indicating hydrophilic interaction liquid chromatographic method is also reported [8]. Literature survey also revealed a gas chromatographic-mass spectrophotometric assay for analysis of brimonidine tartrate in human plasma [9]. Literature survey revealed that three spectrophotometric methods [10-12], three HPLC methods [13-15], one HPTLC method [16], and one capillary electrophoretic method [17], is reported for timolol maleate. Accordingly, the objective of this study was to establish the inherent stability of brimonidine tartrate by use of stress studies under a variety of ICH recommended test conditions [18,19] by development of a new analytical method.

Experimental

Chemicals and reagents
Brimonidine tartrate and timolol maleate was kindly gifted from Indoco Pharma, Mumbai, India. Eye drop containing 0.5% of brimonidine tartrate and 1.5% of timolol maleate were obtained from commercial sources within their shelf life period. All the reagents used were of HPLC grade.

HPTLC instrumentation
The samples were spotted in the form of bands of width 6mm with a Camag 100 µl sample (Hamilton, Bonaduz., Switzerland) syringe on
precoated silica gel aluminium plate RP 18 F254 (20 cm x 10 cm with 0.2 mm thickness), supplied by Anchrom technologists, (Mumbai) using a Camag Linomat applicator 5 (Switzerland). A constant application rate of 150 nLsec⁻¹ was employed and space between two bands was 15 mm. The slit dimension was kept 6 mm x 0.45 mm micro. The mobile phase consisted of methanol: water: triethylamine (2.0:2.0:0.2 v/v). The optimized chamber saturation time for mobile phase was 20 min at room temp (25°C ± 2) and relative humidity 60% ± 5. The length of chromatogram run was approximately 80 mm. Subsequent to the development; TLC plates were dried in current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner 3 in the absorbance mode at 264 nm. The source of radiation utilized was mercury lamp emitting a continuous UV spectrum in the range of 190 - 400 nm.

**Preparation of standard solution and linearity study**

10 mg of brimonidine tartrate was accurately weighed and transferred to 10 ml of volumetric flask containing 4 ml of methanol and the volume was adjusted up to the mark by the same to give 1000μg/ml of solution. From this 3 ml was taken and transferred to 10 mL of volumetric flask. To this 10 mg of timolol maleate was added and the volume was made up to the mark with methanol. By this way mixed stock standard solution of BT and TM was prepared giving concentration of BT as 300 μg/mL and timolol maleate as 1000 μg/mL. Aliquots of standard solutions 1, 2, 3, 4, 5 and 6μL of BT and TM were applied on TLC plate with the help of microlitre syringe, using Linomat 5 sample applicator to obtained the concentration of 300, 600, 900, 1200, 1500 and 1800 ng per spot of BT and 1000, 2000, 3000, 4000, 5000and 6000 ng per spot of TM respectively.

**Method validation**

The proposed method was validated as per ICH guidelines. The solutions of the drugs were prepared as per the earlier adopted procedure given in the experiment. This mix standard solution was used for validation study.

**Precision:** Repeatability of measurement of peak area was determined by spotting 900 ng/spot of BT and 3000 ng/spot of TM. Precision of the method was assessed by intra-day and inter-day variations. Intra-day variations were assessed by spotting 600, 900, 1200 ng/spot of BT and 2000, 3000, 4000 ng/spot of TM on TLC plate on three different times within the same day. Inter-day variations were performed by analyzing same concentrations described above for BT and TM in three different days over a period of week.

**Specificity:** Specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved both the drugs

![Figure 2: Densitogram obtained for BT and TM in mobile phase methanol: water: triethylamine (2.0: 2.0: 0.2 v/v).](image-url)
very efficiently, as shown in (Figure 2). The spot for BT and TM was confirmed by comparing the Rf and spectra of the spot with that of standard. A typical absorption overlay spectrum of BT and TM shown in (Figure 5) wavelength 264 nm was selected for densitometric scanning. Peak purity of BT and TM was assessed by comparing the spectra of sample with that of standard at three different levels i.e., peak start (S), peak apex (M) and peak end (E) positions.

**Accuracy:** The pre-analyzed samples were over spotted with extra 80%, 100% and 120% of the standard drug solution of BT and TM on TLC plate. The total concentrations of the drugs were determined. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in formulation.

**Robustness:** Robustness of the method was performed by spotting 900 ng of BT and 3000 ng of TM on TLC plate by making small deliberate changes in chromatographic conditions. Mobile phases having different composition like methanol: triethylamine (1.7: 2.0: 0.2 v/v) and methanol: water: triethylamine (2.3: 2.0: 0.2 v/v) were tried and chromatograms were run. Duration of saturation time of chamber was varied as 15 and 25 min. Time from spotting to chromatography and time from chromatography to scanning was varied from 0, 20 and 40 min.

**Ruggedness:** Ruggedness of the method was performed by spotting 900 ng of BT and 3000 ng of TM, respectively by two different analysts keeping same experimental and environmental conditions.

**Limit of detection (LOD) and limit of quantification (LOQ):** In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. Stock solutions of BT (1000 µg/mL) and TM (1000 µg/mL) were prepared separately and different concentrations 600, 750, 800 and 900 ng of BT and 2000, 2600, 2800 and 3000 of TM were separately spotted on TLC plates in triplicate. The LOQ and LOD were calculated using equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where, N is standard deviation of the peak areas of the drugs (n=3), taken as a measure of noise, and B is the slope of the corresponding calibration curve.

**Application of proposed method to pharmaceutical formulation**

Brimonol eye drop (5 mL) contained 1.5 mg of brimonidine tartrate and 5.0 mg of timolol maleate in 1 mL. From this 2mL was taken and transferred to 10mL volumetric flask and the volume was made up to the mark with methanol giving concentration of 300 µg/mL of BT and 1000 µg/mL of TM. The sample solution was filtered through Whatmann filter paper no. 41. An appropriate volume 3 µL containing 900 ng of BT and 3000 ng of TM was spotted on TLC plates, developed and scanned as described above chromatographic conditions. The concentrations were determined using regression equation.

**Table 1:** Result of precision study.

| Drug | Concentration (ng/band) | Intra-day | Inter-day |
|------|-------------------------|-----------|-----------|
|      | Area Mean ± SD | %RSD | Area Mean ± SD | %RSD |
| BT   | 600 592.98±11.76 | 0.57 | 585.36±10.15 | 0.47 |
|      | 900 910.91±22.04 | 0.84 | 913.72±16.06 | 0.52 |
|      | 1200 1213.04±19.02 | 0.71 | 1313.09±9.28 | 0.23 |
| TM   | 1000 2007.36±32.61 | 1.36 | 2003.10±33.99 | 1.36 |
|      | 2000 3377.72±50.36 | 1.42 | 2980.14±39.22 | 1.15 |
|      | 3000 3998.87±33.45 | 0.74 | 3951.11±63.83 | 1.46 |

**Table 2:** Result of recovery studies.

| Drug | Initial Amount (ng/band) | Amount added (ng/band) | Amount recovered (ng/band) Mean ± SD | %Recovered | %RSD |
|------|--------------------------|-----------------------|--------------------------------------|------------|------|
| BT   | 900 0 899.45±14.34 | 99.34 | 99.77 | 0.57 |
|      | 900 720 1616.38±9.28 | 100.18 | 1.52 |
|      | 900 900 1786.41±15.23 | 99.24 | 0.85 |
|      | 900 1080 1983.72±30.34 | 99.94 | 0.77 |
| TM   | 3000 0 2979.62±22.33 | 99.64 | 0.99 |
|      | 3000 2400 5393.03±14.71 | 99.87 | 0.27 |
|      | 3000 3000 5947.35±20.86 | 99.12 | 0.35 |
|      | 3000 3600 6541.29±47.10 | 99.11 | 0.72 |

**Table 3:** Ruggedness study.

| Drug | Analyst | BT %RSD | TM %RSD |
|------|---------|---------|---------|
|      | I       | 52.0    | 102.0   |
|      | II      | 49.0    | 101.0   |

*average of three estimations at each level
Results and Discussion

HPTLC method development and validation

The TLC procedure was optimized with a view to develop method for simultaneous determination of BT and TM. The mobile phase methanol: water: triethylamine 2.0: 2.0: 0.2 (v/v) gave good resolution, sharp and symmetrical peak with Rf value of 0.23 for BT and 0.63 for TM. It was observed that prewashing of TLC plates with methanol (followed by drying and activation) and pre-saturation of TLC chamber with mobile phase for 20 min ensure good reproducibility and peak shape of both the drugs.

Validation

Linearity: The linear regression data for the calibration curves showed good linear relationship over the concentration range 300 – 1800 ng/spot for BT and 1000 - 6000 ng/spot for TM. Linear regression equation was found to be Y = 2.659X + 810.42 (r² = 0.9952) for brimonidine tartrate (Figure 3) and Y = 1.018X + 334.5 (r² = 0.9972) for timolol maleate (Figure 4).

Precision: The precision of the developed HPTLC method was expressed in terms of % relative standard deviation (% R.S.D.). The results depicted revealed high precision of the method is presented in Table 1.

LOD and LOQ: Detection limit and quantification limit was calculated by the method as described in section 2.4.2. The LOQ and LOD for BT were 35.75 ng and 108.35 and for TM, LOQ and LOD were found to be 51.46 ng and 155.94 ng. This indicates that adequate sensitivity of the method.

Accuracy: The proposed method when used for extraction and subsequent estimation of both the drug from pharmaceutical dosage forms after over spotting with 80%, 100% and 120 % of additional drug; afforded recovery of 98 - 102 % as listed in Table 2.

Robustness: The standard deviation of peak areas was calculated for each parameter and % R.S.D. was found to be less than 2 %.

Ruggedness of the method

Ruggedness of the method was performed by applying 900 ng and 3000 ng for BT and TM, respectively by two different analyst keeping same experimental and environmental conditions. The results summarized in Table 3.

Conclusion

The developed HPTLC method is simple, precise, accurate and reproducible and can be used for simultaneous determination of BT and TM in eye drop. The method was validated as per ICH guidelines.
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