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An Integrated Pharmaceutical Analysis Utilizing Dual HPTLC For Simultaneous Estimation Of Brinzolamide And Brimonidine Tartrate In Bulk And Ophthalmic Formulation

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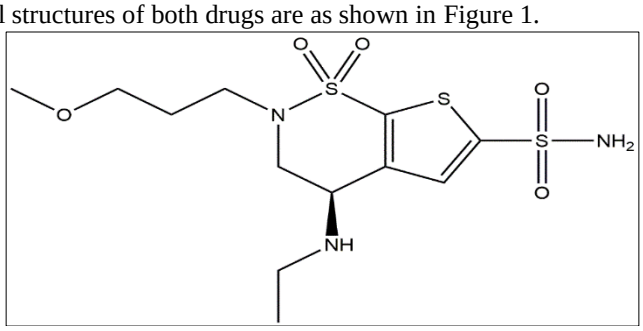
Abstract
Background: Brinzolamide and Brimonidine Tartrate are Anti-glaucoma agents. Both are new active substances which are useful only for topical use in the treatment of glaucoma.
Methods: Two novel, simple, accurate and reliable High-Performance Thin-Layer Chromatography (HPTLC) methods for simultaneous analysis of Brinzolamide (BRZ) and Brimonidine Tartrate (BT) in bulk and ophthalmic formulation. The first method was normal phase chromatography performed on Precoated silica gel F254 TLC plate, with Dichloroethane: Methanol: Triethylamine (4.2: 0.3:0.5, v/v/v) as a mobile phase. The second method was reversed phase chromatography on RP-18 Silica gel F254S TLC plates, with Acetonitrile: water (3.8:1.2, v/v) as mobile phase. HPTLC quantitation of BRZ and BT was done at 260 nm.
Results: The HPTLC method resulted into a compact and well resolved band for BRZ and BT at retention factor (R_f) of 0.39 ± 0.01 and 0.61 ± 0.01 for NP-HPTLC and 0.63 ± 0.01 and 0.43 ± 0.01 RP-HPTLC, respectively. The quantitation by HPTLC method was performed over the concentration range of 300 - 1800 ng/band for BRZ and 100 - 600 ng/band for BT with regression coefficient: r^2> 0.99 for both methods.
Conclusion: A new, precise, and accurate HPTLC method was developed and validated as per ICH guidelines.

Key words: Brinzolamide, Brimonidine Tartrate, NP-HPTLC, RP-HPTLC and validation.

Introduction

Brinzolamide (BRZ), is chemically (R)-4-(ethyl amino)-3, 4-dihydro-2-(3-methoxypropyl)-2 H-thieno [3, 2-e]-1, 2-thiazine-6-sulphonamide1, 1-dioxane. It is a highly specific, non-competitive, reversible carbonic anhydrase II (CA-II) inhibitor indicated to reduce elevated intraocular pressure (IOP) in patients with ocular hypertension or open-angle glaucoma. It is a new active substance which is useful only for topical use in the treatment of glaucoma [1]. The drug is official in Indian Pharmacopoeia [2] and United State pharmacopoeia [3].

Brimonidine Tartrate (BT), 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)quinoxalin-6-amine;(2R,3R)-2,3-dihydroxybutane dioic acid. It is alpha-2 adrenoreceptor agonist. It is used to lower IOP in patients with open-angle glaucoma or ocular hypertension. The IOP lowering efficacy of Brimonidine tartrate ophthalmic solution diminishes over time in some patients. This loss of effect appears with a variable time of onset each patient and should be closely monitored [1-3]. The chemical structures of both drugs are as shown in Figure 1.



a)

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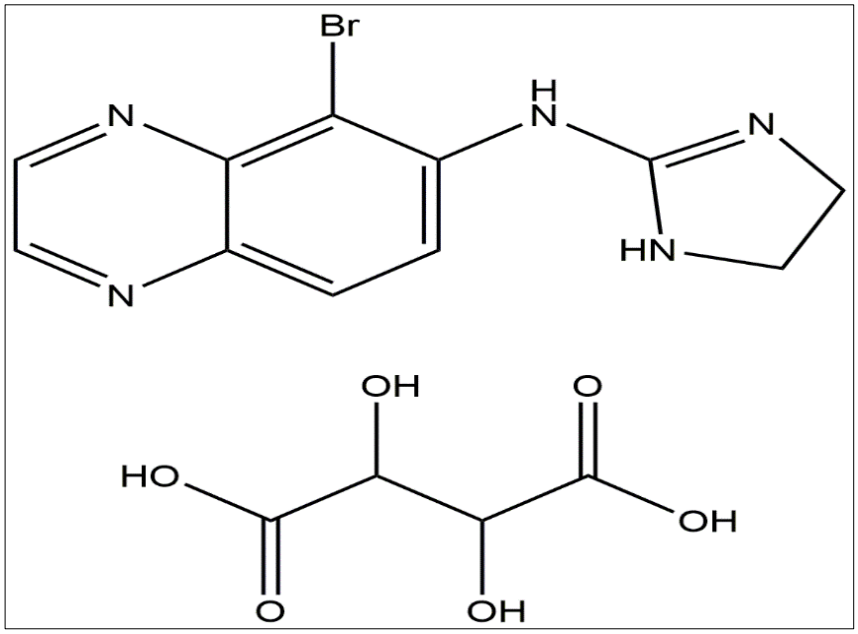

b)

Figure 1: Chemical Structure of a) Brinzolamide and b) Brimonidine Tartrate

Several analytical methods have been developed to estimate the BRZ and BT alone or with Timolol, such as UV [4,5], RP-HPLC [6,-8], HPTLC [9-11], LC-MS/MS [12], stability indicating method by UPLC [13]. Also novel LC-QTOP-MS/MS method for quantitation of BRZ in dried blood spot was reported in literature [14].
The accessible data illustrate no literature for simultaneous study of Brinzolamide and Brimonidine Tartrate by high-performance thin-layer chromatography (HPTLC). Hence, proposed investigation was intended to establish a novel; simple, efficient, rapid and economical NP-HPTLC and RP-HPTLC method for simultaneous analysis of BRZ and BT in bulk and ophthalmic formulation. The proposed method was developed and validated as per the International Conference on Harmonization (ICH) guidelines [15,16].

Experimental

Drugs and Reagents

Pure Brinzolamide (BRZ) were procured from Amneal Pharmaceutical Ltd. Ahmadabad (Prahladnagar). Brimonidine tartrate (BT) was obtained as a gift sample from Alembic Pharmaceutical Ltd., Vadodara, Gujarat, India. Methanol was purchased from Merck Ltd., Worli, and Mumbai, India. Dichloroethane (HPLC), Methanol (HPLC), Acetonitrile (HPLC), Triethylamine (HPLC) was purchased from Merck (India) Ltd., Worli, and Mumbai, India. Ophthalmic solution (Synca®) was purchased from Indian market, containing BRZ (10 mg/mL), BT (2 mg/mL). Double distilled water of HPLC grade was prepared by distillation system.

Instrumentation

Camag TLC system (Muttenz, Switzerland) involves of Camag Linomat 5 sample applicator, Hamilton syringe (100 µL), Camag TLC scanner 3, Camag winCATS software (version 1.3.0), Camag twin trough chamber (20 x 10 and 10 x 10 cm) and ultrasonicator; ENERTECH Electronics Pvt. Ltd., India were utilizing throughout the analysis. Normal-phase chromatography separation was performed on 20 cm × 10 cm silica gel F254 HPTLC plates, while reversed phase chromatography was performed on 10 cm × 10 cm RP-18 F254S HPTLC plates having 200 µm thicknesses (E. Merck, Mumbai, India). Before to use, NP-HPTLC and RP-HPTLC plates washed with methanol and dried in oven at 110˚C for 5 mins. The quantification was carried out using TLC scanner 3 (Camag) installed with win CATS software, Drug sample applied on HPTLC plates using Linomat 5 applicator (Camag) under nitrogen gas flow. Plate were development in a twin trough chamber, with Dichloroethane: Methanol: Triethylamine (4.2: 0.3:0.5, *v/v/v*) and Acetonitrile: water (3.8:1.2, *v/v)* as mobile phase for NP and RP-HPTLC respectively, chamber saturation time 20 min at room temp (28˚C ± 2) for both methods. The scanning was done using densitometric TLC scanner 3 at 260 nm in absorbance and reflectance mode with deuterium lamp emitting a regular UV-spectrum between 190 nm - 800 nm.

Preparation of stock standard solution

Stock standard solution was prepared by weighing 100 mg of BRZ and 20 mg of BT. Weighed powder was transferred into separate volumetric flask of 100 mL dissolved it and diluted to mark with methanol to obtain concentration 1000 µg/mL of BRZ and 200 µg/mL of BT.

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Preparation sample solution

An accurately measured volume of ophthalmic formulation (SYNCA® 5 mL, Label claim: BRZ 10 mg/mL and 2 mg/mL) equivalent to 10 mg of BRZ and 2 mg of BT was transfer into 10 ml volumetric flask containing 5 ml of methanol and further solution was made up to the mark using methanol, filtered using 0.45 µm filter (Mill filter, Milford, MA). From filtrate, 1 ml of solution was transferred into 10 mL volumetric flask and volume was made up to mark with methanol to obtain the concentration 100 µg/mL BRZ and 20 µg/mL of BT. A fixed volume of 10 µL solutions was applied on NP-HPTLC and RP-HPTLC plates and subjected to proposed methods and the amount of BRZ and BT were determined.

Method validation

The proposed NP and RP-HPTLC methods was validated as per ICH guidelines to secure them for linearity, precision, selectivity, sensitivity, robustness, accuracy and specificity.

Optimization of mobile phase

To accomplish high resolution and reproducible peaks, there are many mobile phase mixtures were initially tested. For NP-HPTLC analysis silica gel F254 HPTLC plates and Dichloroethane: Methanol: Triethylamine (4.2: 0.3:0.5, v/v/v) as mobile phase were selected as optimum. A sharp and well resolved peak was obtained for BRZ and BT at Rf of 0.39 ± 0.01 and 0.61 ± 0.01 when the chamber was saturated with mobile phase for 20 min at room temperature. In RP-HPTLC analysis RP18 silica gel F254S plates with Acetonitrile: water (3.8:1.2, v/v) as mobile phase were chosen as optimum. The wavelength of 260 nm was selected to be optimal for the highest sensitivity. A sharp and well resolved peak was obtained for BRZ and BT at Rf of 0.43 ± 0.01 and 0.64 ± 0.01 when the chamber was saturated with mobile phase for 20 min at room temperature.

Linearity and calibration curve

From stock solution, an appropriate volume 0.3-1.8 mL of BRZ was transferred into the series of 10 mL volumetric flask and volume made up to the mark with methanol. From each volumetric flask a volume of 10 µL was applied on NP-HPTLC and RP-HPTLC plate to obtain series of concentration 300 - 1800 ng/band.

From the stock solution, an appropriate volume 0.5 - 3 mL of BT was transferred into series of 10 mL volumetric flask and volume made up to the mark with methanol and fixed volume of 10 µL was over spotted on the NP-HPTLC and RP-HPTLC plates to obtain concentration 100 - 600 ng/band of BT respectively. The linear calibration curve was constructed by plotting peak-areas against drug quantity per band.

The linear calibration curve was plot between a peak area against concentration of BRZ and BT. The linearity of the methods was good, which is generally considered as evidence of acceptable fit was shown in Figure 2 and 3 for both methods. The HPTLC standard chromatogram of BRZ and BT is shown in Figure 4 for both methods.

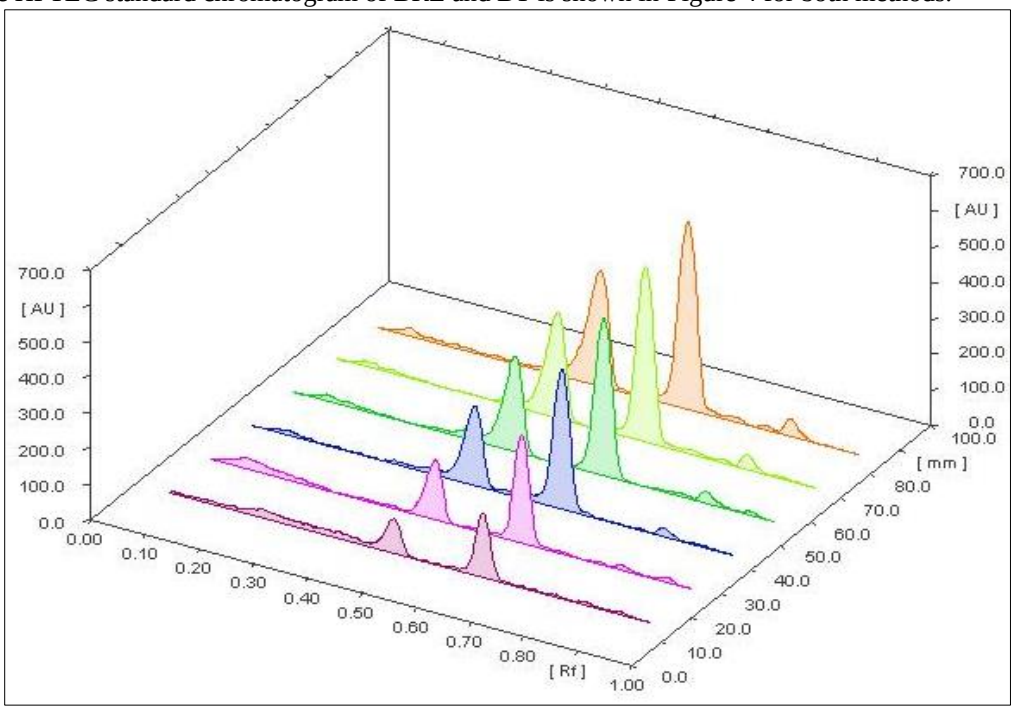

Figure 3: Linearity studies for Brinzolamide and Brimonidine Tartrate in Acetonitrile: water (3.8:1.2, v/v) as mobile phase during RP-HPTLC analysis.

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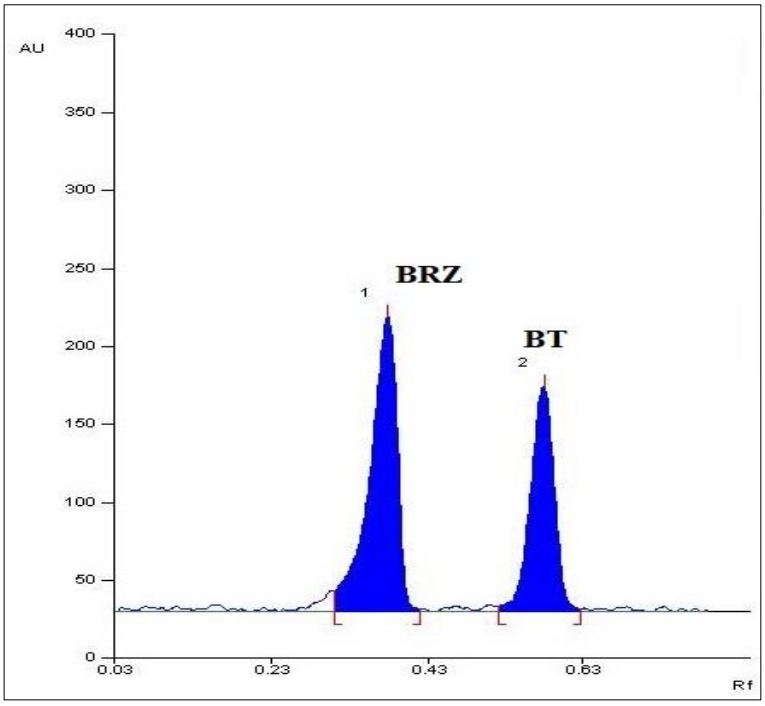
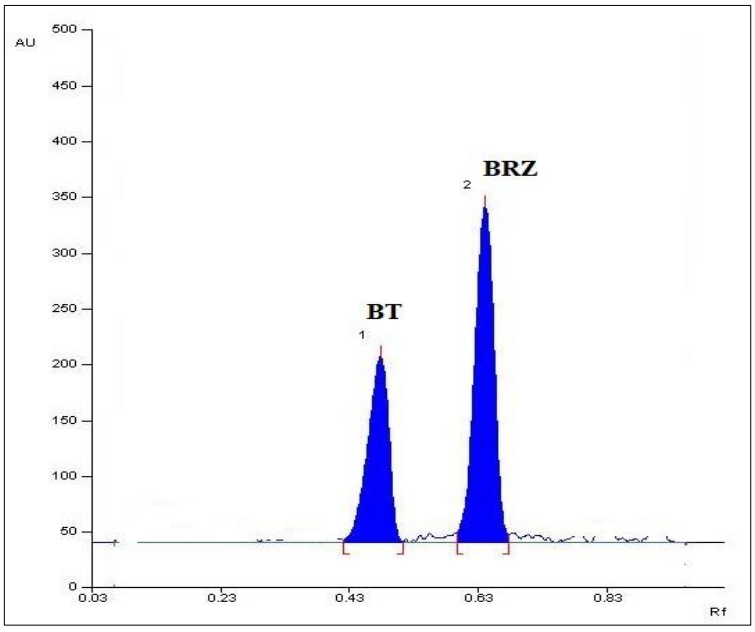


NP-HPTLC Chromatogram of Brinzolamide and Brimonidine Tartrate



RP-HPTLC Chromatogram of Brinzolamide and Brimonidine Tartrate

Figure 4: Standard chromatogram of Brinzolamide and Brimonidine Tartrate

Precision of the method

For repeatability, intra-day and inter-day precision of the methods were estimated for three different amounts of BRZ and BT. All the measurements were repeated six times for each concentration.

DL and QL

The Detection Limit (DL) and Quantification Limit (QL) of BRZ and BT were obtained experimentally, by inspecting the signal-to-noise ratio as described by the International Conference for Harmonisation guidelines Q2 (R1) (ICH). For sensitivity studies different volume of stock solution in the range 300-600 ng/band and 100-200 ng/band for BRZ and BT respectively was spotted on TLC plate. The procedure was repeated in triplicate. DL and QL were calculated by the use equation DL = 3.3 x N/B and QL = 10 x N/B, where 'N' is standard deviation of the peak areas of the drugs (n=3),

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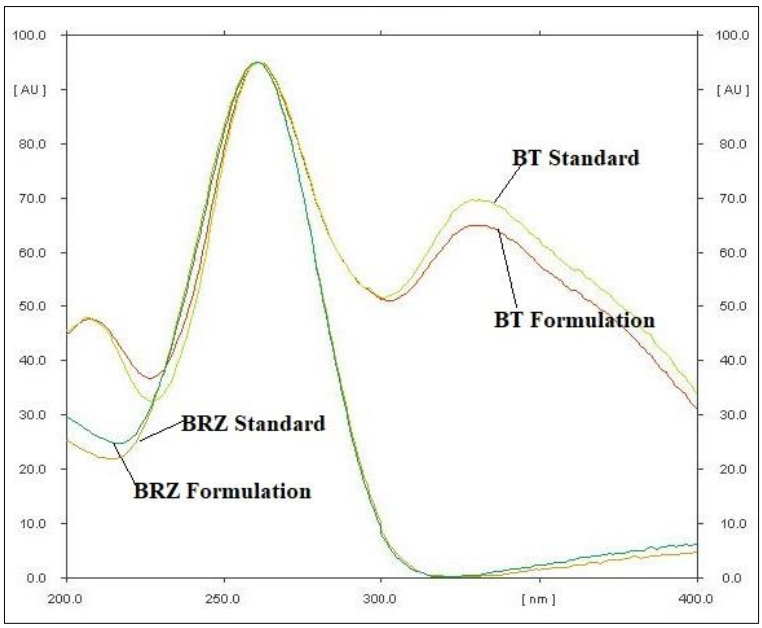
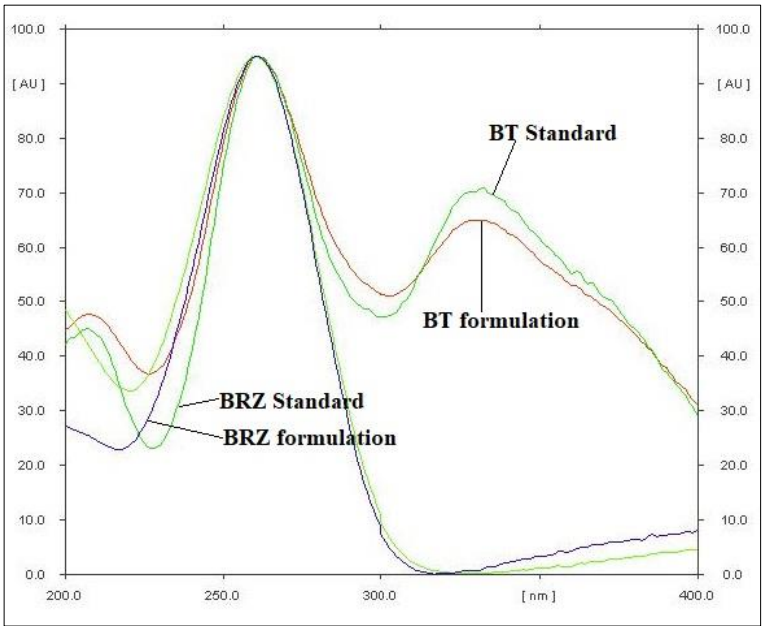
taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve. The results were recorded for both the methods.

Specificity

A typical absorption spectrum of BRZ and BT was shown in Figure 5 the peak - purity of BRZ and BT was hardened by correlating the spectra of BRZ and BT added to laboratory at the peakstart (S), peak - apex (A) and at the peak - end (E) positions. Correlation between these spectra indicated purity of BRZ and BT peak {correlation r (S, M) = 0.999, 0.989, r (M, E) = 0.999, 0.998 for NP-HPTLC and r (S, M) = 0.994, 0.998, r (M, E) = 0.996, 0.997} RP-HPTLC for developed both method analysis.



Peak-purity spectrum for NP-HPTLC



Peak-purity spectrum for RP-HPTLC

Figure 5: Peak-purity spectrum of BRZ and BT

Accuracy

Recovery study were execute at three different levels i.e. 80, 100 and 120 %. To the pre-analyzed sample solutions, a known amount of mixed drug standard solutions of BRZ and BT were over spotted at three different levels.

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Robustness
Robustness study is to determine by of its tendency to confront deliberated changes in method parameter. The robustness was demonstrated by performing test method in normal situations and each altered condition mentioned below. In proposed investigation, four parameters such as mobile phase composition, development distance, duration of saturation was analyzing for robustness of method.

Ruggedness
Ruggedness of HPTLC method was performed at a concentration of 1000 ng/band BRZ and 200 ng/band BT. Methods were creating to be rugged when analysis was accomplished by two different analysts under the unchanged experimental and ecological condition.

Results and discussion
BRZ and BT showed a good correlation coefficient for all methods was shown in Table 1, the given concentration range 300-1800 ng/band BRZ and 100-600 ng/band BT. The % amounts predicted from ophthalmic formulation show that there is no interference from excipients present in it. The percentage amounts of BRZ and BT assessed from ophthalmic formulation using for both methods are shown in Table 2. The DL and QL for BRZ were found to be 24.84 ng and 75.30 ng, respectively while for BT the DL and QL was found to be 15.83 ng and 14.97 ng, respectively in NP-HPTLC method. The DL and QL for BRZ were found to be 31.27 ng and 94.75 ng, respectively while for BT the DL and QL was found to be 9.51 ng and 28.25 ng, respectively in RP-HPTLC method. The intra-day and inter-day precision was carried out by executing three replicates of three different concentrations 1000, 1250 and 1500 ng/ band of BRZ and 200, 250 and 500 ng/band of BT revealed % RSD less than 2 indicating that both methods was precise. The percentage recovery of BRZ and BT at three concentration levels 80, 100, and 120 % was established in the range of 99.21-100.28%, implementing equitableness of method to perform routine drug analysis. Ruggedness of HPTLC methods was executed two different analysts and % RSD less than 2 indicated that a method was rugged. The robustness of the current investigation was assessed by accomplished little but deliberate changes in thin layer chromatographic conditions. Furthermore, the method was found to be specific as the excipients did not show any obstruction at the retention factor of BRZ and BT. In this investigation, the chromatographic parameters superintend were retention factor and peak area of both drugs. The results of analysis of robustness study are as shown in Table 3, where % RSD less than 2 designated that the both methods is robust, and is not influenced by small changes in chromatographic conditions. The results of validation parameters are summarized in Table 3.

Table 1: Linearity with Correlation Coefficient

Parameters	**BRZ**		**BT**	
	NP-HPTLC	**RP-HPTLC**	**NP-HPTLC**	**RP-HPTLC**
Linearity [ng/band]	300-1800	300-1800	100-600	100-600
Slope	5.1403	4.8066	9.2425	14.24
Intercept	471.42	1353.3	508.01	24.871
Correlation Coefficient	0.999	0.994	0.989	0.998

Table 2: Analysis of Ophthalmic Formulation

	NP-HPTLC		**RP-HPTLC**	
Drugs	**%Amount found**	**%RSD**	**%Amount found**	**%RSD**
BRZ	101.38	1.01	100.06	0.22
BT	99.37	1.32	99.95	0.50

Table 3: Summary of Validation Parameters

Parameters	**(NP-HPTLC)**		**(RP-HPLC)**	
	BRZ	**BT**	**BRZ**	**BT**
Recovery [% RSD] [n = 3]	0.75 – 0.89	0.89 – 1.81	0.27–0.97	0.32 – 0.58
Ruggedness [% RSD]				
Analyst I [n = 6]	1.18	1.28	0.45	0.25
Analyst II [n = 6]	1.76	1.92	0.25	0.67

Precision [% RSD]				
Inter-Day [n = 6]	0.10 – 0.53	0.70 – 1.82	1.59–1.86	0.34 – 0.74
Intra-Day [n = 6]	0.14 – 0.33	0.21 – 1.80	0.75–1.41	0.92 – 1.43
Repeatability [n = 6]	1.22	0.87	0.41	0.16
Robustness Study [% RSD]				
Mobile phase volume	1.65	1.56	1.21	1.28
Mobile phase composition	1.75	1.13	1.36	1.31
Development distance	1.46	1.75	1.25	1.55
Duration of saturation	0.70	0.73	0.75	0.78

Conclusion

Another, basic, and delicate HPTLC technique has been effectively developed and validated for determination of BRZ and BT in bulk and ophthalmic formulation. In general two techniques were established for quantitative analysis of BRZ and BT in bulk and ophthalmic formulation using NP-HPTLC and RP-HPTLC. Validated method was simple, precise and rugged. Further, the method is found to be accurate and sensitive. The percent recovery in formulation confirms that the excipients commenced in the formulation have no interfering in the determination. Along these lines, proposed techniques can routinely be executed for estimation of BRZ and BT in bulk and ophthalmic formulation.

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Conflict of interest: Authors has no conflict of interest

Abbreviation: BRZ: Brinzolamide, BT: Brimonidine Tartrate

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