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Simultaneous Determination of Azelastine hydrochloride and Benzalkonium chloride by RP-HPLC Method in their Ophthalmic Solution

MEM Hassouna[1]*, MM Abdelrahman[2] and MA Mohamed[3]

[1]*Department of Chemistry, Faculty of Science, Beni-Suef University, Egypt*

[2]*Department of Analytical Chemistry, Faculty of Pharmacy, Beni-Suef University, Egypt*

[3]*EPCI part of HIKMA group, Pharmaceutical Company, Beni-Suef, Egypt*

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***Corresponding author:** MEM Hassouna, Department of Chemistry, Faculty of Science, Beni-Suef University, 62514 Beni-Suef, Egypt, Tel: +2 01223861504; Email: mhassouna47@hotmail.com; Mohamed.hassona@science.bsu.edu.eg

Abstract

A simple, specific, precise, accurate, and stability-indicating RP-HPLC method is developed and validated for the determination of azelastine hydrochloride (AZH) and benzalkonium chloride (BAC) in eye drops formulations. RP-HPLC method was performed on the Thermo CPS CN column (150 mm X 4.6 mm, 5 µm particle size, using buffer solution of pH 4.5 containing 50 mM potassium dihydrogen phosphate and 5.7 mM hexane sulfonate:acetonitrile (50:50 v/v) as the mobile phase at a flow rate of 1.5 ml/min, UV detection at 212 nm and run time of 7.5 min . This method is validated according to ICH guidelines and USP requirements for new methods, which include accuracy, precision, selectivity, LOD, LOQ, robustness, ruggedness, linearity and range. Linear relationships were obtained in the ranges of 6.25-50 µg/mL and 5.0-50 µg/mL for AZH and BAC, respectively, with significantly different R_t values of 2.254, 3.432 and 3.946 min for AZH and homologs of BAC (C12, C14) with correlation coefficients of 0.9988 and 0.9983, respectively (Figure 1).

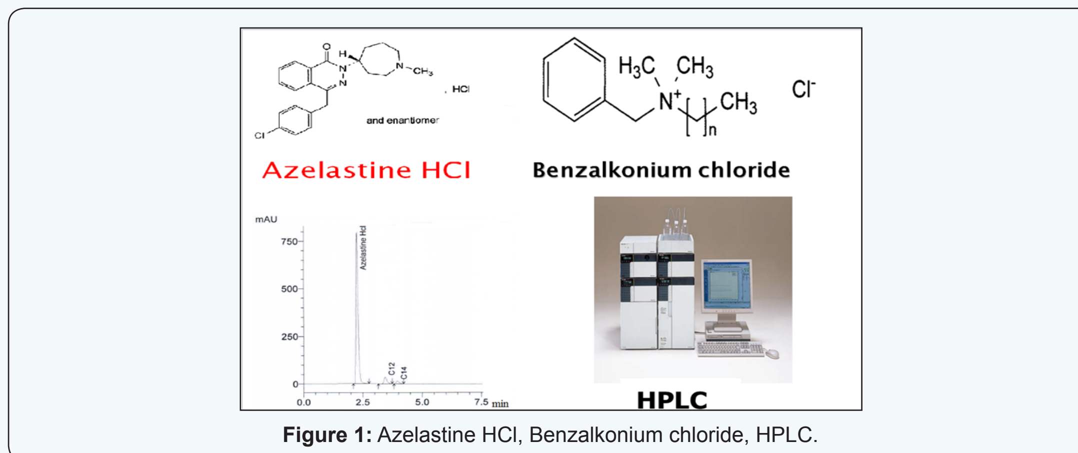

Figure 1: Azelastine HCl, Benzalkonium chloride, HPLC.

Keywords: Azelastine hydrochloride; Benzalkonium chloride; Ophthalmic solution; RP-HPLC

Abbreviations: AZH: Azelastine Hydrochloride; BAC: Benzalkonium Chloride; BP: British Pharmacopeia; EP: European Pharmacopeia; USP: United States Pharmacopeia; TLC: Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography; EPCI: Egyptian Pharmaceutical and Chemical Industry; ACN: Acetonitrile; LOQ: Limit of Quantification; ED: Eye Drops; ICH: International Conference on Harmonization

Introduction

Azelastine hydrochloride (AZH); is chemically known as *4-(4-Chlorobenzyl)-2- [(4RS)-1-methylhexahydro-1H-azepin-4-yl] phthalazin-1(2H)-one hydrochloride* [1,2]. AZH occurs as a white or almost white, crystalline powder. AZH is a potent, second generation, selective, histamine antagonist (histamine-H1-receptor antagonist) used as first line therapy of mild

intermittent, moderate/severe intermittent and mild persistent rhinitis (new classification system for rhinitis). AZH has been formulated both as a nasal spray and as eye drops. Azelastine eye drops are indicated for the local treatment of seasonal and perennial allergic conjunctivitis [3,4]. Benzalkonium chloride (BAC); chemically known as mixture of *alkylbenzyldimethyl ammonium chlorides*, the alkyl groups mainly having chain lengths of C12, C14 and C16. [1,2]. BAC is a frequently used preservative in eye drops; typical concentrations range from 0.004% to 0.01%. Stronger concentrations can be caustic and cause irreversible damage to the corneal endothelium [5]. Azelastine hydrochloride is official in British Pharmacopeia (BP) [1] and European Pharmacopeia (EP) [6], both of them includes potentiometric titration for estimation of AZH. BAC is official in BP [1], EP [6] and United States Pharmacopeia (USP) [7], which include potentiometric titration and HPLC method for its estimation. The combination of these two drugs is not official in any pharmacopoeia.

It is still a limited number of analytical methods that are reported for the determination of azelastine hydrochloride including colorimetric and spectrophotometric [8-12], thin layer chromatography (TLC) [13-16], capillary electrophoresis [17,18], high performance liquid chromatography (HPLC) [19-25], electrochemical methods [26,27] and thermal analysis [28] have been developed for the estimation of AZH individually or in dosage forms. BAC applications are extremely wide, ranging from its use as a disinfectant in formulations to microbial prevention in the oilfield service industry. In preserved drug formulation, BAC is commonly used as a preservative [7], thus a variety of methods have been developed for its determination including spectrophotometric [29-31], TLC [32] and capillary electrophoresis [33-35]. Chromatographic methods have been extensively applied for its determination, GC [36] and HPLC [37-53]. According to the best of our knowledge there is no specific analytical method for the simultaneous determination of both drugs in ophthalmic solution. The main aim of this study is to develop a simple, sensitive, short retention times and accurate RP-HPLC method for the simultaneous determination of AZH and BAC in pure, bulk and dosage forms.

Materials and methods

Chemicals and Reagents

Standards: Standard samples of AZH and BAC (50% solution) were kindly supplied by Egyptian Pharmaceutical and Chemical Industry (EPCI) Company, a part of HIKMA group, Beni-Suef, Egypt with claimed purity of 99.8% and 96.9%, respectively according to manufacturer certificates of analysis.

Pharmaceutical dosage form: Azelast® 0.05% ED (Eye Drops) (Batch No. 1460086) were manufactured by EPCI Company part of HIKMA group, Beni-Suef, Egypt with Mfg. date 05-2014 and Exp. date 05-2016. Each 1 mL is claimed to contain 0.5 mg of AZH and 0.1 mg of BAC.

Chemicals: Acetonitrile HPLC-grade (ACN), potassium dihydrogen phosphate analytical grade, water HPLC grade and 1-Sodium hexane sulfonate were procured from (scharlau, Spain).

Instrumentation

HPLC system (Shimadzu LC SPD 20A) with a detector (dual wavelength), equipped with a binary pump, Autosampler, oven CTO-20A/20AC with temperature range (10-85°C), LC Solution software. HPLC system (Agilent 1260 Infinity, Germany) instrument was equipped with an Agilent 1260 Infinity preparative pump (G1361A), Agilent 1260 Infinity DAD detector VL (G131SD), Agilent 1260 Infinity Thermostated column compartment (G1316A) and Agilent 1260 Infinity preparative Autosampler (G2260A). UV-1800 double beam UV-Visible spectrophotometer (shimadzu-Japan) with highest resolution. Its spectral bandwidth is (1 nm from 190-1100 nm range) was used for all absorbance measurements. Matched with 1 cm quartz cells. Perform data analysis by software (UV-Probe 2.5.2).

UV absorption spectra of 20 µg/mL of each of AZH and BAC using water as blank have been scanned. Their corresponding maxima are illustrated in (Figure 2).

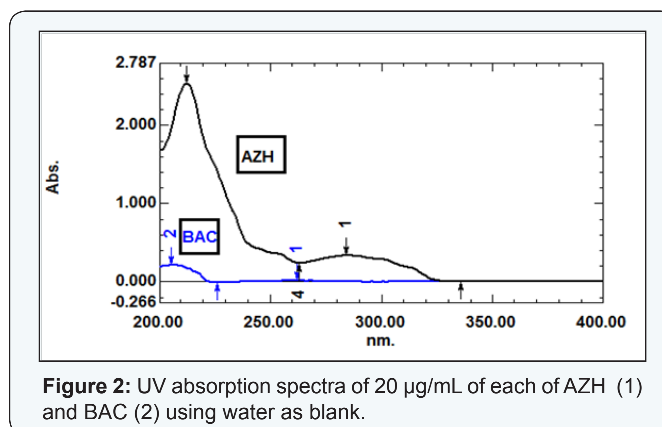

Figure 2: UV absorption spectra of 20 µg/mL of each of AZH (1) and BAC (2) using water as blank.

Mobile phase preparation: ACN: Buffer (50:50)

i. Acetonitrile

ii. Buffer pH 4.5: Accurately weigh 6.8 gm potassium dihydrogen phosphate and 1.167 gm Hexane sulfonate and adjust the pH to 4.5 with orthophosphoric acid solution. Make up to 1000 mL with distilled water. Filter and degas mixtures of (A) acetonitrile and (B) buffer (50:50) through 0.45 µ membrane filter under vacuum pump.

HPLC Chromatographic Conditions: Chromatographic separation was performed on column Thermo Scientific CPS HYPERSIL L10 CN (150 X 4.6 mm i.d, 5 µm particle size) (USA). The following conditions have been applied: a mobile phase mixture of phosphate buffer and acetonitrile in the ratio of 50:50% v/v at ambient temperature, flow rate of 1.5 mL/min, UV detection at 212 nm, injection volume of 20 µL and run time of 7.5 min.

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Preparation of Standard Solution

Stock solutions of Azelastine HCl and Benzalkonium chloride: Transfer 100 mg of the standard Azelastine HCl into 100 mL volumetric flask. Add about 70 mL diluent, sonicate for 10 min till complete dissolution then complete to volume with the diluent. Filter through suitable syringe filter. This solution contains AZH ≡ 1000 µg/mL. Similarly, transfer 100 mg of the standard B into 100 mL volumetric flask. Add about 70 mL diluent, sonicate for 10 min till complete dissolution then complete to volume with diluent. Filter through suitable syringe filter. This solution contains BAC ≡ 1000 µg/mL.

Calibration curves Construction: Transfer accurately aliquots of AZH and BAC equivalent to 6.25-50 and 5-50 µg/mL, respectively from their stock solutions into two 100 mL volumetric flasks, add ≈70 mL of water HPLC grade and sonicate to mix. Complete to the mark with HPLC grade water and mix. Triplicate 20 µL injections were made for each concentration maintaining the flow rate at 1.5 mL/min and the effluents were UV-scanned at 212 nm. The chromatographic separation was performed following the above stated conditions. The chromatograms were recorded, the peak areas of AZH and BAC were determined, the calibration curves relating the obtained integrated peak areas and the corresponding concentrations were constructed and the regression equations were performed.

Authentic Prepared Mixture

N.B.: Due to the low concentration of BAC in dosage form, where the concentration of AZH is five folds greater than BAC, which leads to the disappearance of BAC peak in the chromatogram, so we prepared equal concentrations of each in the mixture to validate their simultaneous determination. Thus, accurately transfer 5.0 mL of AZH and 5.0 mL of BAC from their stock solutions into 100 mL volumetric flask, add 70 mL of water HPLC grade and sonicate to mix. Complete to the mark with water and mix. (Conc. of each of AZH and BAC ≡ 50 µg/mL). The obtained chromatogram is shown in (Figure 3).

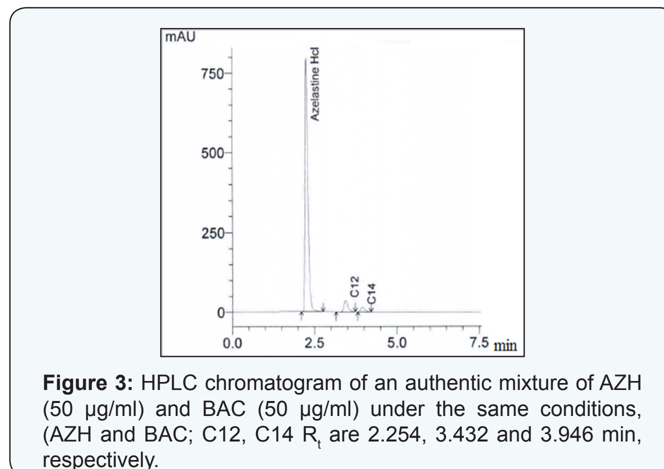

Figure 3: HPLC chromatogram of an authentic mixture of AZH (50 µg/ml) and BAC (50 µg/ml) under the same conditions, (AZH and BAC; C12, C14 R_t are 2.254, 3.432 and 3.946 min, respectively.

Application to pharmaceutical formulation (Azelast 0.05% Eye Drops): Shake and pour the contents of five bottles and their water rinses (each bottle contains 5 ml) of Azelast 0.05% Eye Drops into 100 mL volumetric flask. Accurately transfer 5.0 mL aliquot into 50 mL volumetric flask. Add about 30 mL of distilled water, sonicate and complete to volume with the water and mix well. Thus, we obtain a conc. of AZH & BAC (50,10 µg/mL) respectively. Filter through 0.45 µm syringe filter and inject into the chromatographic system. The standard addition technique has been carried out to assess the validity of the method by spiking the pharmaceutical formulation with known amount of standard solution of AZH and BAC. The recovery of the added standards was then calculated after applying the proposed methods.

Results

The chromatogram obtained at retention times 2.254, 3.432 and 3.946 min, respectively for AZH and homologs of BAC (C12, C14) shows no peak interferences.

Discussion

Table 1: Comparison between the proposed and Rao's methods for the determination of AZH and BAC.

Parameter	Present Method			Rao's Method		
	AZH	BAC		AZH	BAC	
Mobile phase	50:50 (50 mM buffer phosphate pH 4.5+ 5.7 mM hexane sulfonate:ACN)			55:45 (50 mM buffer phosphate:ACN)		
Column used	Thermo CPS CN column (150 mm X 4.6 mm, 5 µm			Waters Spherisorb CN (250 mm x 4.6 mm, 5 µm)		
Flow rate	1.5 ml/min			1.0 ml/min		
Wave length	212 nm			215 nm		
Retention time	2.254 min	C_{12}	C_{14}	6.647 min	C_{12}	C_{14}
		3.432 min	3.946 min		10.175 min	12.179 min
Run time	7.5 min			20 min		
Ranges (µg/mL)	6.25-50	5.0-50		39.2 - 67.2	2.8 - 4.8	

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Correlation coefficient	0.9994	0.9992	0.997	0.9997
Accuracy (Mean±RSD)	101.55±1.01	100.72±1.00	101.35±1.47	101.56±1.37
Intermediate Precision	0.828	0.890	0.06	1.36
LOD[a] (μg/mL)	1.25	0.92	NA	NA
LOQ[a] (μg/mL)	2.41	3.79	NA	NA

[a]Limit of detection (3.3 × SD/Slope) and limit of quantitation (10 × SD/Slope).

A single publication [23] that related the simultaneous determination of both AZH and BAC besides fluticasone propionate and phenyl ethyl alcohol by RP-HPLC method in nasal sprays has been published in 2010; this was performed on a 250 mm x 4.6 mm, 5- μm particle size, Waters Spherisorb CN column using a mobile phase composed of 55:45 (v/v) mixture of buffer and acetonitrile, UV detection at 215 nm and flow rate of 1.0 mL/min. The present method was performed on a much shorter column, the Thermo CPS CN column (150 mm X 4.6 mm, 5 μm particle size, using buffer solution of pH 4.5 containing a completely different mobile phase, its composition is illustrated above at a relatively slower flow rate of 1.5 mL/min, UV detection at 212 nm and run time of 7.5 min. The conditions of the present method succeeded in achieving linear relationships in the ranges of 6.25-50 μg/mL and 5.0-50 μg/mL, respectively, compared with corresponding ranges of 39.2 - 67.2 μg/mL for azelastine hydrochloride and 2.8 - 4.8 μg/mL for benzalkonium chloride [23]. The dynamic range of the latter (BAC) is quite limited. Also, significantly different and short R_t values of 2.254, 3.432 and 3.946 min for AZH and homologs of BAC (C12, C14) were achieved with correlation coefficients of 0.9988 and 0.9983, respectively compared to corresponding R_t values of 6.647 & 10.175, 12.179 min with correlation coefficients of 0.997 and 0.9997, respectively [23]. In the present work, heat stress studies and photo degradation have been performed. A comparison between the two methods is recorded in (Table 1).

Methods development and optimization

Many systems of different compositions and ratios were tried including: methanol:water (70:30, v/v), Acetonitrile (ACN): water (70:30, v/v). Buffer phosphate pH (6.3):ACN(30:70, v/v) led to a splitted peak, while the buffer composed of phosphate pH(4.5):ACN (30:70, v/v) gave bad separations. On trying buffer phosphate pH(4.5) + hexane sulfonate:ACN (55:45, v/v) mixture, it was found that the presence of hexane sulfonate in the developing system is essential for the separation and improving tailing of peaks, but still eluted late. Buffer phosphate pH (4.5) + hexane sulfonate:ACN (50:50, v/v) has been tried, (0.8, 1.0,1.2 and 1.5 mL/min) flow rates have been tried, scanning wavelengths (208, 210, 212, 264 and 284 nm) have also, been tried also tried. Preliminary studies involved the trial of C18 reversed-phase columns. The best developing system was buffer phosphate pH (4.5) + hexane sulfonate:ACN (50:50, v/v) applying a flow rate of 1.5 mL/min at a wavelength of 212 nm using column L10 Thermo CN (150 mm X 4.6 mm i.d., 5 μm). This selected developing system allows good separation between the studied components with good R_t values without tailing of the separated bands and good theoretical plates.

Method validation

The method was validated, in accordance with ICH guidelines (ICH Q2R1), for system suitability, precision, accuracy, linearity, specificity, ruggedness, robustness, LOD and LOQ [54].

I. **Linearity and range:** The linearity of the proposed methods was obtained in the concentration range (6.25-50.0 μg/mL) for AZH and (5.0-50.0 μg/mL) for BAC. Calibration curves were constructed by plotting peak areas against the corresponding concentration. The obtained coefficients of regression were 0.9988 and 0.9983, respectively for AZH and BAC, while the obtained slopes were 89358.23 and 5981.41, respectively. Linearity results are shown in (Table 2).

II. **Repeatability:** Repeatability is conducted using 6 replicates of the standard solution of the compound under study. The system is precise as the relative standard deviation RSD ≤2%. The high precision of the method is shown in (Table 2).

Table 2: Regression and validation parameters of the proposed HPLC method for determination of AZH and BAC.

Parameter	AZH	BAC
Linear range (μg/mL)	6.25-50	5-50
Slope	89358.23	5981.41
Intercept	-53173.7844	763.4819
Correlation coefficient	0.9994	0.9992
Precision	0.828	0.890
LOD[a] (μg/mL)	1.25	0.92
LOQ[a] (μg/mL)	3.79	2.41

[a]Limit of detection (3.3 × SD/Slope) and limit of quantitation (10 × SD/Slope).

III. **Detection and Quantitation limits:** These approaches are based on the standard deviation of the blank. Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses. Where limit of detection (LOD) = 3.3 × SD/slope and limit of quantification (LOQ) =10 × SD/slope, (Table 2).

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IV. Accuracy and recovery: Accuracy of the proposed methods was calculated depending on the percentage recoveries of pure samples of the studied drugs. The accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true one. Accuracy should be assessed using a number of nine determinations over a minimum of three concentrations with average recovery percent ranging between 97% and 103% of spiked analyte amount for AZH and BAC. These results are shown in (Table 3). Accuracy was further assessed by applying the standard addition technique to Azelast 0.05% Eye Drops (ED), where good recoveries were obtained revealing that there were no interference from excipients (Table 4).

Table 3: Data of Accuracy for Azelastine HCl and Benzalkonium chloride.

Azelastine HCl	Azelastine HCl			Benzalkonium chloride	Benzalkonium chloride		
Standard Solution(µg/mL)	µg/mL (Injected)	µg/mL (found)	Recovery%	Spiked level (µg/mL)	µg/mL (injected)	µg/mL (found)	Recovery%
12.5	12.5	12.56	100.48%	10	10	10.22	102.28%
	12.5	12.57	100.62%		10	10.19	101.92%
	12.5	12.50	100.05%		10	9.90	99.08%
25	25	25.64	102.58%	20	20	20.10	100.51%
	25	25.71	102.85%		20	20.28	101.41%
	25	25.69	102.77%		20	20.13	100.65%
37.5	37.5	38.03	101.43%	30	30	30.10	100.34%
	37.5	38.13	101.69%		30	30.16	100.53%
	37.5	38.05	101.48%		30	29.93	99.79%
Accuracy (Mean)	101.55			Accuracy (Mean)	100.72		

Table 4: Determination of AZH and BAC in their pharmaceutical formulation by the proposed HPLC method and application of standard addition technique.

Pharmaceutical formulation	Added(µg/mL)		Recovery %		Found %	
	AZH	BAC	AZH	BAC	AZH	BAC
Azelast 0.05% Eye Drops	7	5	98.73	97.97	100.20±2.381	101.97±0.142
AZH, 0.5 mg(claimed)	10	10	101.16	102.79		
BAC, 0.1 mg(claimed)	15	15	101.47	100.38		
Mean ± RSD			100.45±1.588	100.76±2.592		

V. Formulation assay: This study is performed by assaying five samples from the finished product with the same concentration prepared from a homogenous sample starting from the first step of analysis procedure to the final one. The result of the assay undertaken yielded 92.4% of the label claim for Azelast ED. The results of the assay indicate that the method is selective for the analysis of Azelast ®ED without interference from excipients used to formulate and produce this ED. The results are displayed in (Table 5).

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Table 5: Assay results for the determination of AZH and BAC in their pharmaceutical formulation by the proposed HPLC method.

Pharmaceutical formulation	Conc.(µg/mL)		Recovery %		limit %	
	AZH	BAC	AZH	BAC	AZH	BAC
Azelast 0.05% Eye Drops	25	20	93.22	112.70	(90 -110)	(80 - 120)
AZH, 0.5 mg(claimed)			95.49	117.54		
BAC, 0.1 mg(claimed)			96.24	117.91		
			94.24	117.43		
			94.50	115.89		
Mean ± RSD			94.74±1.229	116.29±1.850		

VI. Intermediate precision (ruggedness): Intermediate precision expresses the within-laboratories variations: different days, different analysts, different equipments, etc. Satisfactory results were obtained and are presented in (Table 6).

Table 6: Ruggedness of the method.

Parameter(%RSD)	AZH	BAC
Intraday	1.685	1.636
Interday	0.526	0.976
Analyst to Analyst	1.158	2.014
Apparatus to Apparatus	1.184	2.669

VII. Robustness: Robustness of an analytical method is a measure of its capacity to remain unaffected by small variations in method parameters. The variables taken into account were the percentage of organic modifier as acetonitrile composition (±5%), pH of the mobile phase (± 0.2), flow rate (±0.1 mL/min) and column temperature change (40°C & 20°C). The low values of the % RSD, as given in (Table 7), indicate the robustness of the proposed methods.

Table 7: Robustness of the method.

Parameter (%RSD)	AZH	BAC
Flow rate change (±0.1 mL/min)	2.470	1.550
pH change of mobile phase (±0.2)	1.924	2.903
Organic (ACN) composition change (±0.5%)	2.972	1.255
Column temperature change(40,20°C)	2.599	1.482

VIII. System suitability: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability was checked by calculating tailing factor (T), column efficiency (N) and resolution (R_s) factors. All calculated parameters were within the acceptable limits indicating good selectivity of the methods and assuring the system performance, (Table 8).

Table 8: System suitability testing parameters of the developed method.

Item	Obtained value		Reference values
	AZH	BAC	
Tailing factor	1.485	1.252	T ≤ 2
Resolution (R_s)	-	10.129	R >2
Injection precision	0.828	0.890	RSD ≤1%
Number of theoretical plates(N)	3820.109	5826.635	N > 2000

Stability of the analytical solution

After preparing the standard solution, part of it was stored in fridge and another part was stored at room temperature. After about 24 hours, these solutions were tested against freshly prepared standard; relative standard deviation should be less than 2%.These results are displayed in (Table 9).

Table 9: Result of stability of analytical solution.

Condition	AZH	BAC*
Fridge	100.4%	95.68%
Room temperature (22°C)	100.3%	94.18%

*Potency of BAC standard= 96.6%.

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Specificity

I. Placebo interference: Method specificity was determined for samples of studied compounds and placebo matrix containing all excipients present in the finished product. No interferences were detected at the retention times of AZH and BAC (C12, C14).

Forced degradation

Forced degradation of the active pharmaceutical ingredient (API) was carried out according to ICH guidelines (ICH, Q2B) in acid, base, oxidation, photo and heat.

I. Photo degradation: The powder of standard AZH was kept under sunlight for 48 hours. From this powder, accurately weigh 25.0 mg and transfer to 100-mL volumetric flask. Add 60-70 mL of distilled water and sonicate to dissolve. Make up to the mark with water and mix well. Dilute 10 mL aliquot from this solution with water to 100 mL. Filter through 0.45 µm membrane filter, reject the first portion then analyze by HPLC. No interference was found at the retention time of AZH and no degradation products appeared after light degradation.

II. Heat degradation: Keep the powder sample of the standard AZH in dry oven at 80ºC for 6 hours. Similarly, weigh 25.0 mg; dissolve in 100-mL volumetric flask. Dilute, filter then analyze by HPLC. No interference was found at the retention time of AZH and no degradation products appeared after heat degradation.

III. Acid degradation: Accurately weigh 25.0 mg of the standard AZH powder and transfer into 100-ml conical flask. Add 60-70 mL of distilled water and sonicate to dissolve, add 10 mL of 0.1 N HCl then keep the acidified solutions in boiling water bath for one hour. Then keep at room temperature till cooling and complete to the mark with water and mix well. Dilute 10 ml aliquot from this solution to 100 ml volumetric flask with water. Filter and analyze by HPLC. No interference was found at the retention time of AZH and no degradation products appeared after acid degradation.

IV. Base degradation: Similarly, weigh 25.0 mg of AZH standard powder and transfer to 100-mL conical flask. Add 60-70 mL of distilled water and sonicate to dissolve, add 10 ml of 0.1 N NaOH then keep the alkaline solution in boiling water bath for one hour, keep at room temperature till cooling. Complete to 100 mL with water and mix well. Dilute 10 mL aliquot from this solution to 100 mL with water. Filter and analyze by HPLC as usual. No interference was found at the retention time of AZH, but its peak area was affected after base degradation. The degradation percent was calculated and displayed in (Table 10).

Table 10: Results of analysis of forced degradation study samples using proposed method, indicating percentage degradation of AZH and BAC.

Test Name		Azelastine HCl			Benzalkonium chloride		
	Effect	Observed t_R	Peak Area	Degradation %	Observed t_R	Peak Area	Degradation %
Test	Without Effect(control)	2.518	2196179	-	4.551	115519	-
	Oxidation Effect	2.533	2080094	5.28	4.572	107630	6.82
	Alkali Effect	2.657	1365682	37.8	4.579	101725	11.94
	Acid Effect	2.457	2276286	0.97	4.534	114459	0.91
	Light Effect (Sun light)	2.534	2188403	0.35	-	-	-
	Heat Effect	2.535	2171644	1.11	4.551	113794	1.49
	Placebo	No peak observed	No area observed	-	No peak observed	No area observed	-

V. H_2O_2 degradation: Accurately weigh 25.0 mg of the standard AZH powder and transfer to 100-mL volumetric flask. Add 60-70 mL of distilled water and sonicate to dissolve, add 15 mL of 3.0% H_2O_2 then keep at room temperature for half an hour, complete to the mark with water and mix well. Dilute 10 mL aliquot of this solution to 100 mL with water. Filter and analyze by HPLC. No interference was found, although there was some degradation products appeared after the H_2O_2 treatment due to the peak of hydrogen peroxide. The degradation percent was calculated and depicted in (Table 10). Thus, forced degradation can be summarized: AZH was found to be stable under heat, light, acidic conditions and is labile under alkaline and oxidation. While, BAC is found to be stable under heat, light conditions and is labile under alkaline, acidic and oxidation. The conditions of degradation are shown in (Table 10). The corresponding chromatogram obtained was shown in (Figure 4), also, peak purity was applied for AZH in (Figure 5).

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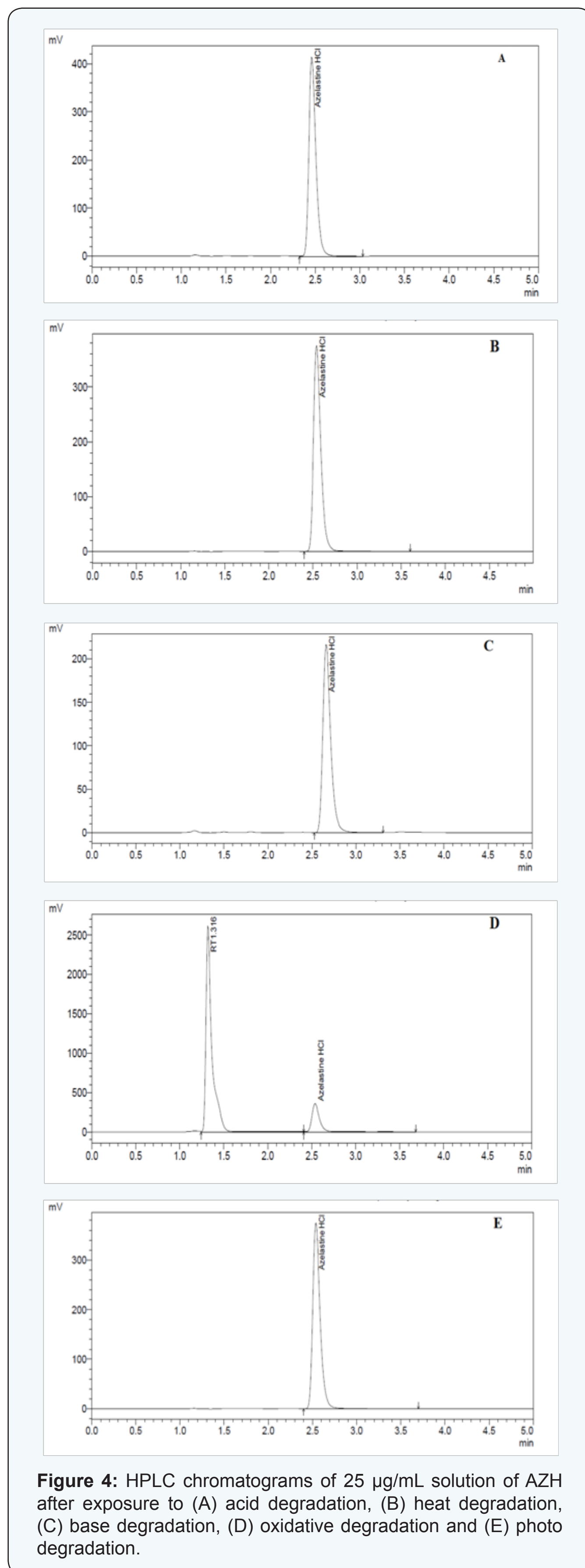

Figure 4: HPLC chromatograms of 25 µg/mL solution of AZH after exposure to (A) acid degradation, (B) heat degradation, (C) base degradation, (D) oxidative degradation and (E) photo degradation.

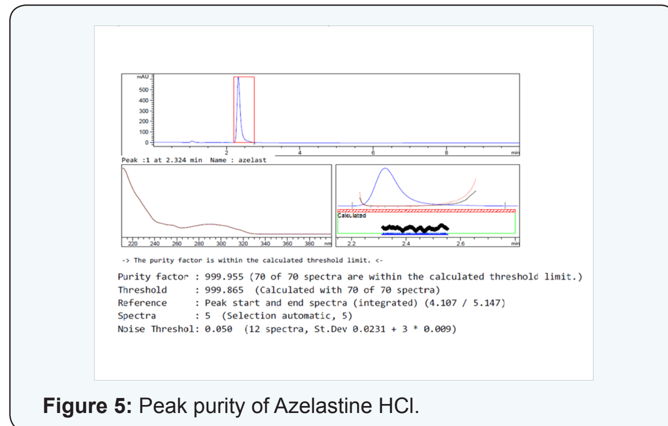

Figure 5: Peak purity of Azelastine HCl.

Conclusion

The proposed RP-HPLC method for the simultaneous estimation of azelastine hydrochloride and benzalkonium chloride in combined ophthalmic solution is simple, precise, specific, highly accurate, less time consuming for analysis, low cost and rapid. The results of stress testing have been undertaken according to the International Conference on Harmonization (ICH) guidelines and revealed that AZH was found to be stable under heat, light, acidic conditions and labile under alkaline ,oxidation. BAC was found to be stable under heat, light conditions and labile under alkaline, acidic and oxidation. Based on the above results, the analytical method is valid, fit for use and can be applied for regular routine analysis and stability study.

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Conflict of Interest

Compliance with Ethical Standards: The authors declare that they have no conflict of interest.

Research involving human participants and/or animals

This article does not contain any studies with human participants or animals performed by any of the authors.

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