World Journal of Applied Chemistry
2017; 2(2): 48-56
http://www.sciencepublishinggroup.com/j/wjac
doi: 10.11648/j.wjac.20170202.12

Determination of Azelastine Hydrochloride and Benzalkonium Chloride in Their Ophthalmic Solution by Different Spectrophotometric Methods

Mohamed E. M. Hassouna[1, *], Maha M. Abdelrahman[2], Mahmoud A. Mohamed[3]

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

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

[3]HIKMA Group, Pharmaceutical Company, Beni-Suef, Egypt

Email address:

mhassouna47@hotmail.com (M. E. M. Hassouna), Mohamed.hassona@science.bsu.edu.eg (M. E. M. Hassouna), maha_m_abdelrahman@yahoo.com (M. M. Abdelrahman), ch.mahmoud88@gmail.com (M. A. Mohamed)

[*]Corresponding author

To cite this article:

Mohamed E. M. Hassouna, Maha M. Abdelrahman, Mahmoud A. Mohamed. Determination of Azelastine Hydrochloride and Benzalkonium Chloride in Their Ophthalmic Solution by Different Spectrophotometric Methods. *World Journal of Applied Chemistry.* Vol. 2, No. 2, 2017, pp. 48-56. doi: 10.11648/j.wjac.20170202.12

Received: February 8, 2017; **Accepted**: February 23, 2017; **Published**: March 10, 2017

Abstract: Three simple, specific, accurate and precise spectrophotometric methods for the determination of a binary mixture of Azelastine hydrochloride (AZH) and Benzalkonium chloride (BAC) in pure and in their Dosage forms. The methods namely; (A) zero order (D^0), (B) ratio difference (RD) and (C)ratio subtraction (RS). In method (A), AZH could be directly determined in the zero order at wave length equals to 284 nm in the range (5.0–50 µg/mL) with good correlation coefficient (0.9994), where no interference from BAC is reported. While BAC was determined by different methods in the range (2.0–30 µg/mL). In method (B) BAC determination depends on dividing the spectrum of the binary mixture by the standard spectrum of 20µg/mL AZH as a divisor, then BAC is determined using the difference between the two wavelengths 262 & 208 nm, we here BAC has absorbances and AZH is constant, the difference = (ΔP262.0-208.0) with good correlation coefficient (0.9994). Finally, in method (C) BAC can be determined by dividing the spectrum of the binary mixture by the standard spectrum of 10 µg/mL AZH as a dvisor, then a constant, which is determined as the mean value of the absorbances in the plateau region (230–300 nm), is subtracted; after multiplication by the divisor we obtain a zero order (D^0) original spectrum of BAC at 208.0 nm with good correlation coefficient (0.9992). Accuracy, recovery and the selectivity of the developed methods are applied on the laboratory prepared mixtures, standard addition technique and pharmaceutical dosage form. The obtained results for the suggested methods are statistically compared with the reported HPLC one using student's-t and F-ratio tests, showing that the two methods are accurate and precise.

Keywords: Azelastine Hydrochloride, Benzalkonium Chloride, Ophthalmic Solution, Zero Order (D^0), Ratio Subtraction Method (RS), Ratio Difference Method (RD)

1. Introduction

Azelastine hydrochloride (AZH); is chemically known as 4-(4-Chlorobenzyl)-2-[(4*RS*)-1-methylhexahydro-1*H*-azepin-4-yl] phthalazin-1(2*H*)-one hydrochloride [1, 2] (Figure1). AZH occurs as a white, almost odorless, crystalline powder with a bitter taste. It has a molecular weight of 418.37. AZH is a potent, second-generation, selective, histamine antagonist (histamine-H1-receptor antagonist). It is an intranasal antihistamine indicated as an appropriate medical treatment for patients suffering from the seasonal allergic rhinitis (SAR) and non allergic vasomotor rhinitis(VMR). Reportedly, this medicament is also used topically in the symptomatic relief of both acute and chronic allergic conditions, [3, 4]. Benzalkonium chloride(BAC); chemically known as mixture of alkylbenzyldimethyl ammonium chlorides, the alkyl groups mainly having chain lengths of C_{12}, C_{14} and C_{16}. [1, 2] (Figure 1). This mixture has a bactericidal effect against Gram-positive and Gram-negative

bacteria, and is widely used as an antimicrobial preservative in topical aqueous pharmaceutical preparations, especially in ophthalmic solutions [5].

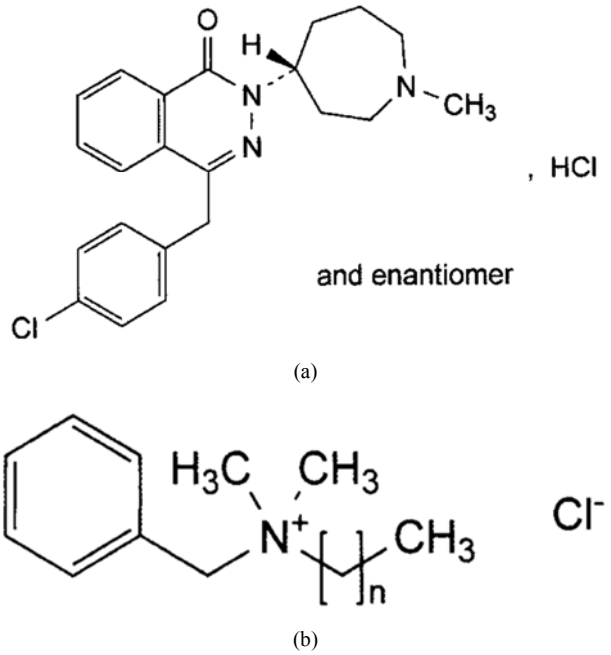

Figure 1. *Chemical structures of (a) Azelastine HCl, (b) Benzalkonium Chloride.*

Azelastine hydrochloride is official in British Pharmacopeia (BP) [1] and European Pharmacopeia (EP) [6], both of them includes potentiometric titration for the estimation of AZH. Benzalkonium chloride is official in BP [1], EP [6] and United States Pharmacopeia (USP) [7], which includes potentiometric titration and HPLC method for the estimation of BAC. The combination of these two drugs is not official in any pharmacopoeia. It is still a limited number of analytical methods that were reported for the determination of Azelastine hydrochloride including colorimetric and spectrophotometric [8-13], thin layer chromatography (TLC) [14-17], capillary electrophoresis [18, 19], high performance liquid chromatography (HPLC) [20–27], electrochemical methods [28, 29] and thermal analysis [30] have been developed for the estimation of AZH individually or in dosage forms.

BAC applications are extremely wide, ranging from being disinfectant in formulations to microbial prevention in the oil field 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 [31-33], TLC [34] and capillary electrophoresis [35-37]. Chromatographic methods have been extensively applied, GC [38] and HPLC [39-57]. Recently, a spectrophotometric method has been published reporting the simultaneous determination of Fluticasone propionate and Azelastine hydrochloride in the presence of pharmaceutical dosage form additives [13].

More recently [58], a stability-indicating RP-HPLC method has been developed and validated for the simultaneous determination of both azelastine hydrochloride (AZH) and benzalkonium chloride (BAC) in laboratory authentic mixtures and in eye drops formulations.

According to the best of our knowledge, there is no spectrophotometric method for the determination of azelastine hydrochloride and benzalkonium chloride in authentic mixtures and in dosage form. Thus, the present study is directed towards the development of such method with the aim of reconsideration of spectrophotometry being simple, sensitive, accurate and available technique for the determination of AZH and BAC in pure and ophthalmic dosage forms versus the sophisticated published methods and to validate them according to ICH guide lines [59].

2. Materials and Methods

2.1. Apparatus

UV-1800 double beam UV-Visible spectrophotometer (Shimadzu-Japan) with highest resolution which spectral band width is 1nm for the spectral range190-1100 nm, was used for all absorbance measurements. Matched with1cm quartz cells. Data analysis is performed by software (UV-Probe 2.5.2).

2.2. Reagents and Materials

Purified water is used as solvent, pure samples of AZH and BAC are kindly supplied by the Egyptian Pharmaceutical and Chemical Industry (EPCI) Pharmaceutical Company part of HIKMA group, Beni-Suef, Egypt with claimed purity of 99.8% and 96.9%, respectively, according to manufacturer certificates of analysis. Azelast® 0.05% Eye Drops ED (Batch No. 1460086) were manufactured by EPCI Pharmaceutical Company part of HIKMA group, Beni-Suef, Egypt. Each 1mL is claimed to contain 0.5 mg of AZH and 0.1mg of BAC.

Preparation of the Standard Solutions.

2.2.1. Stock Solutions of Azelastine Hydrochloride and Benzalkonium Chloride (1000µg/mL)

100 mg of each of AZH & BAC standards are weighed accurately, transferred into100 mL volumetric flask, 70 mL of solvent are added and sonicated to dissolve. The flask is made up to the mark with the same solvent and mixed well.

2.2.2. Working Standard Solutions of Azelastine Hydrochloride and Benzalkonium Chloride (100 µg/mL)

Accurately transfer 10 mL of AZH and BAC from their stock solutions in two 100 mL volumetric flasks, add 70 mL of the solvent and sonicate to dissolve. Make up to the mark with the same solvent and mixed well.

2.2.3. Laboratory Prepared Mixture

Prepare mixtures of AZH and BAC containing different ratios from their working standard solutions (100 µg/mL) into a series of 10 mL volumetric flasks. Make up to the mark with the same solvent.

The concentration of AZH and BAC are calculated from the corresponding regression equations describing the linearity for each method.

2.2.4. Dosage Form Preparations (Azelast 0.05% Eye Drops)

Shake and empty five eye droppers into 100 mL graduated cylinder and mix them together so as to obtain a conc. of AZH & BAC (0.5, 0.1 mg/mL), respectively. The concentration and recovery of dosage form were calculated from the corresponding regression equations relating the linearity for each method. Standard addition technique is carried out to prove the validity of the method by spiking the pharmaceutical dosage form with known amount of the standard solution of each drug alternatively. The recovery of the added standards was, then, calculated after applying the proposed methods.

2.3. Construction of Calibration Curves

Different aliquots of AZH and BAC equivalent to 2–30 and 5-50 µg/mL, respectively, were separately transferred from their respective working solutions (100 µg/mL) into two separate series of 10 mL volumetric flasks, and the volumes were made up to the mark with distilled water. The calibration curves relating the obtained absorbances to the corresponding concentrations are constructed and the regression equations are performed. Zero order absorption spectra of AZH and BAC are scanned in the range of 200-400 nm (Figure 2).

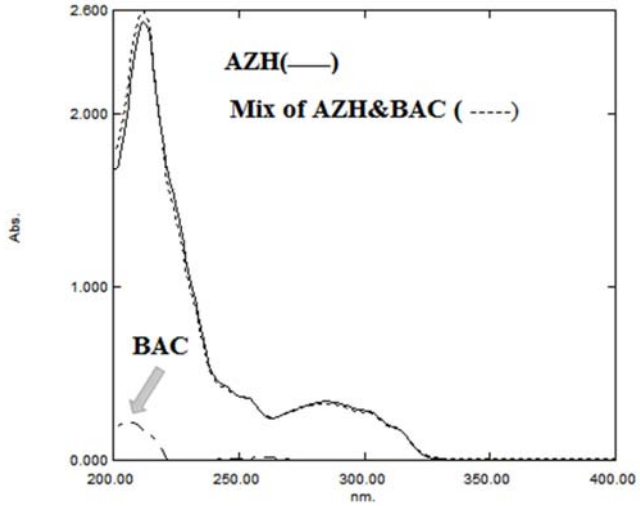

Figure 2. *UV absorption spectra of 20µg/mL of each of AZH(——), BAC(——)and mix of 20µg/mL of AZH & BAC(----)using water as a blank.*

2.4. Direct Spectrophotometric Method (D^0)

Azelastine hydrochloride gives maximum absorbance at a wavelength equals to 284 nm, the calibration curve relating the obtained absorbances and the corresponding concentrations in the range 2.0-30 µg/mL is constructed and the regression equation is calculated (Figure 3).

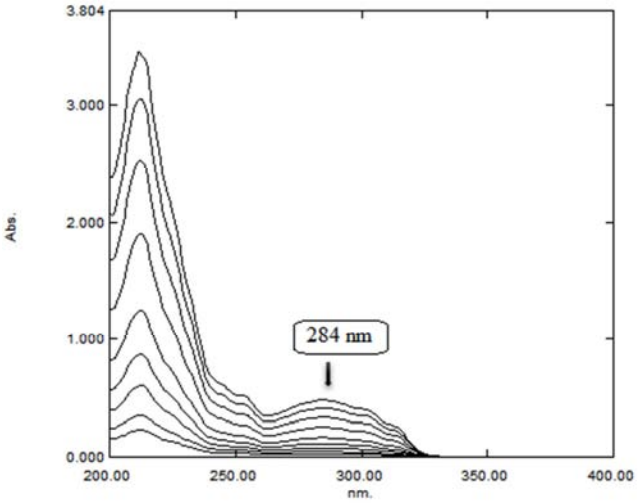

Figure 3. *Zero order absorption spectra of (2-30) µg/mL of AZH using water as a blank.*

2.5. Ratio Difference Method

The scanned spectra of different laboratory authentic mixtures AZH and BAC are divided by the spectrum of 20 µg/mL AZH as a divisor. The ratio spectra are recorded. Calibration curves are constructed by plotting the difference between the amplitude of the ratio difference at 262 nm & 208 nm for BAC, versus the corresponding concentrations and the regression equations are computed.

2.6. Ratio Subtracting Method

The stored spectra of the mixture of the two drugs AZH and BAC were divided by the spectrum of the concentration 10 µg/mL AZH as a divisor then a constant value, which is determined in the plateau region from 230–300 nm is subtracted, a new spectrum will be obtained. If we multiply the new spectrum by the spectrum of 10 µg/mL AZH as original divisor, we can obtain the zero order (D^0) spectrum of BAC. The calibration curve relating the absorbances of the zero order absorption spectra at 208.0 nm versus the corresponding concentrations of BAC is drawn and the regression equations are computed.

2.7. Application to Pharmaceutical Formulation

Different concentrations of AZH and BAC in their ophthalmic solution are determined by spiking each of them by known concentration of pure standard drug then, subtracting the spiked amount. The procedure of each method mentioned above was then followed. The validity of the method was further confirmed by applying the standard addition technique.

3. Results and Discussion

The purpose of this work is to reconsider the ability of spectrophotometry as a simple, facile, sensitive, accurate, precise and available technique for the determination of binary mixture of two important drugs, viz., AZH and BAC

in their pure form and ophthalmic solution. Also, achieves the accuracy and recovery for laboratory prepared mixture. The standard addition technique is applied for each method. Overlapping was occurred in the zero order in the case of the mixture AZH and BAC and couldn't be resolved neither by first nor the second derivative techniques, especially in the determination of BAC. Hence, the search for other methods that can be applied in such cases. (Figure2). For convenience, each method will be discussed separately.

3.1. Ratio Difference Method

The important role of the ratio difference spectrophotometric method (RD) is measuring the values of ratio difference at two wavelengths and also, alternates the derivative steps besides the improvement of the signal to noise ratio. Ratio difference spectrophotometric method(RD) is applied to solve the overlapped spectra of BAC at two wavelengths 262 & 208 nm, the difference = (*ΔP*262.0-208.0) which give absorbances while the ratio difference of AZH is constant (Figure 4). All concentrations of BAC (5-50 µg/mL) are divided by the spectrum of AZH (20 µg/mL) (Figure 4).

Calibration curves were constructed by plotting the difference between the amplitude ratio difference at *ΔP*262 nm & 208 nm for BAC, versus the corresponding concentrations and the regression equations are computed.

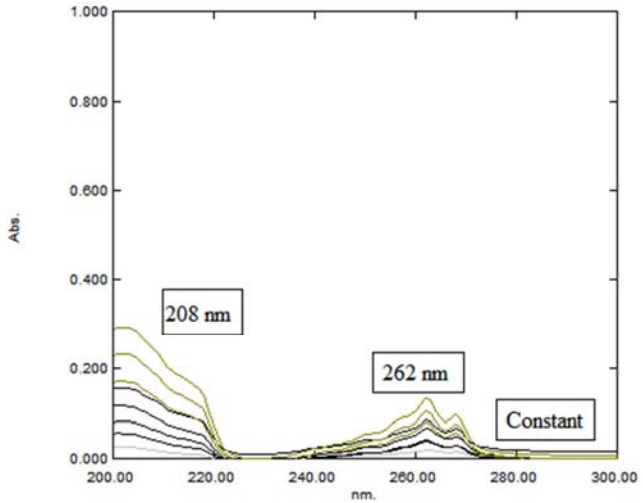

Figure 4. *Ratio spectra of (5-50 µg/mL) of BAC using spectrum of 20 µg/mL of AZH as a divisor.*

3.2. Ratio Subtraction Method

The principle of this method is that the prepared mixture is divided by suitable concentration of the two drugs as a divisor (the spectrum of 10 µg/mL AZH). The obtained results from the division will give a new spectrum. A constant value which is determined as the mean value of the absorbances in the plateau region (230–300 nm) is subtracted from the ratio spectra, after that the obtained spectrum is multiplied by the divisor. Finally, we can obtain a new zero order spectrum of BAC (Figure 5). The calibration curve is constructed by plotting the absorbance values of BAC at 208 nm of the latter obtained zero order spectrum, versus the corresponding concentrations and the regression equation is computed.

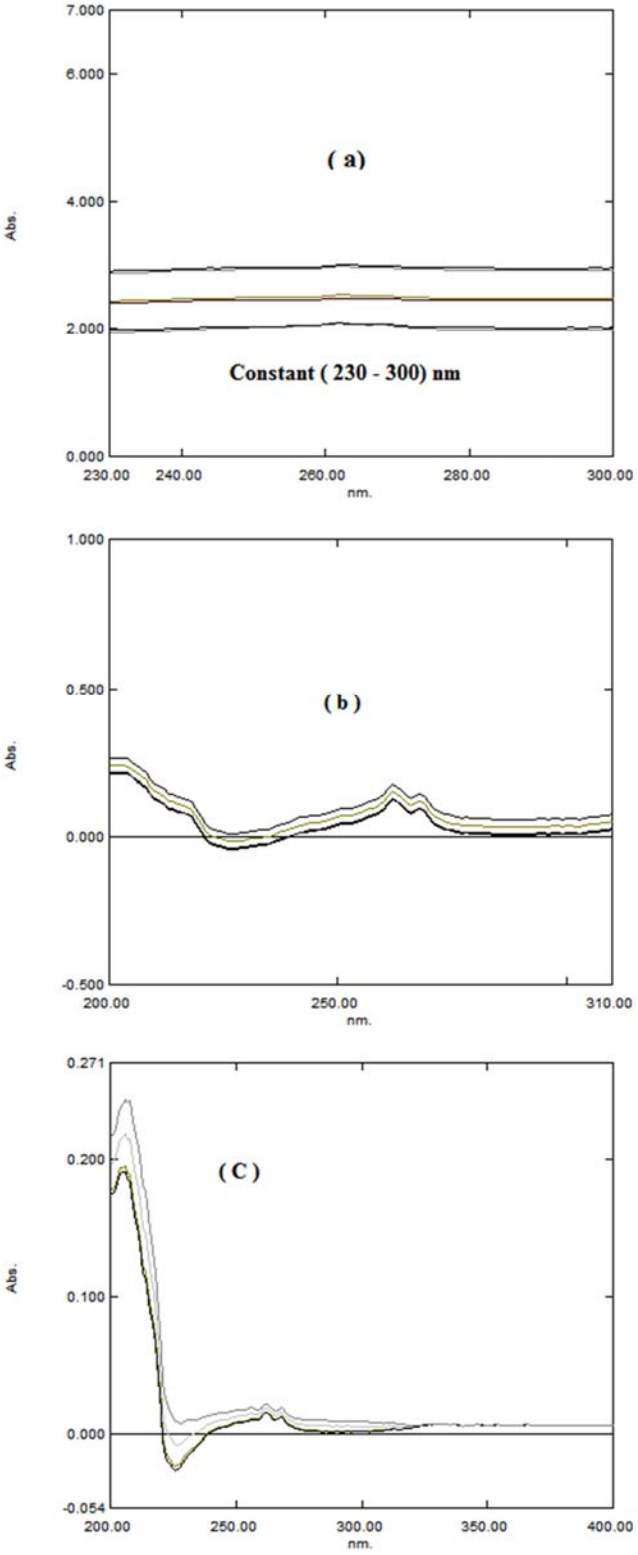

Figure 5. *(a)Ratio spectra of mixtures of AZH and BAC using 10 µg/mL of AZH as a divisor, (b)Ratio spectra after subtraction of constant, (c)New spectrum of BAC in zero order.*

3.3. Methods Development and Optimization

Different trials have been spent for the sake of optimizing the different parameters that affecting the accuracy and recovery of the developed methods such as: solvent, wavelengths and divisors…etc. On performing the first derivative for the analysis of the studied mixture, it gave good results for AZH, but overlapping occurred with BAC, so, we tried the use of second derivative and first derivative ratio, but, unfortunately, no satisfactory results ha ve been obtained. In the ratio subtraction and ratio difference, many solvents have been tried such as methanol, 0.1N HCl, 0.1N NaOH and water; experimentally, water behaved as the best solvent. Also, regarding the wavelengths, different wavelengths have been tested to achieve good accuracy and recovery for dosage form, standard addition and laboratory prepared mixtures, so we select 284 nm for AZH in the zero order, 208 nm for BAC in the ratio subtraction method and 262 nm for BAC in the ratio difference method. On the other hand, selection of the divisor's concentration is very critical in order to get the best result, thus, we chose the concentration 20 µg/mL AZH as a divisor in the ratio difference and 10 µg/mL AZH in the ratio subtraction methods.

3.4. Validation of the Analytical Method

The method was validated, in accordance with ICH guide lines [59].

3.4.1. Linearity and Range

The linearity of the proposed methods is obtained in the concentration range (2.0-30.0 µg/mL) for azelastine hydrochloride and (5.0-50.0 µg/mL) for benzalkonium chloride. The obtained results of correlation coefficients, slope and intercept indicate the good linearity. Linearity results are shown in Table 1.

3.4.2. Repeatability

Expresses within-laboratories variations: The intraday RSD % (n=3), average of three concentrations (10,20 and 30 µg/mL) for each of AZH and BAC respectively, are repeated three times within the day. Good results are obtained as shown in Table 1.

Table 1. *Regression and validation parameters of the proposed method for determination of AZH and BAC.*

Parameter Methods	AZH D^0	BAC	
		RD	RS
Wavelengths	284 nm	(262–208) nm	208 nm
Range (µg/mL)	2-30	5-50	5-50
Slope	0.0164	0.0075	0.0114
Intercept	-0.0006	0.0024	-0.0131
Correlation coefficient	0.9994	0.9994	0.9992
Repeatability	0.10	0.92	0.78
LOD[a] (µg/mL)	0.64	1.16	1.32
LOQ[a] (µg/mL)	1.86	3.53	4.02
*RSD %[b]	0.139	0.215	0.175
*RSD %[c]	0.145	0.445	0.280

[a]Limit of detection (3.3 × σ/Slope) and limit of quantitation(10 × σ/Slope). *RSD %[b] & *RSD %[c]: the intra-day and inter-day respectively(n=3) relative standard deviation of concentrations (10, 20,30 µg/mL).

3.4.3. Intermediate Precision (Ruggedness)

The inter day RSD % (n=3), average of three concentrations (10,20 and 30 µg/mL) for AZH and BAC respectively, are repeated three times in three successive days. Good results were obtained and presented in Table1.

3.4.4. Detection and Quantitation Limits

These approaches are based on the Standard Deviation of the Response and the Slope. A specific calibration curve should be studied using samples, containing an analyte in the range of LOD and LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. LOD=3.3 × σ/slope and LOQ =10 × σ/slope, where σ=the standard deviation of the response as shown in Table1.

3.4.5. Accuracy and Recovery

Accuracy of the proposed methods is calculated as the percentage recoveries of pure samples of the studied drugs. Accuracy is assessed using three different concentrations covering the specified range (i.e., three concentrations and three replicates). Concentrations are calculated from the corresponding regression equations. The mean % recoveries for AZH and BAC are between 98.0% to 102.0% and are shown inTable 2.

Table 2. *Data of Accuracy for Azelastine HCl and Benzalkonium chloride.*

Benzalkonium chloride Standard Solution(µg/mL)	BAC					AzelastineHCl Standard Solution (µg/mL)	AZH D^0		
	RD			RS					
	µg/mL(taken)	µg/mL(found)	Recovery%	µg/mL(found)	Recovery%		µg/mL(taken)	µg/mL(found)	Recovery%
	12	11.91	99.28%	12.16	101.37%		5.0	5.09	101.82%
12	12	12.18	101.49%	11.99	99.90%	5.0	5.0	5.04	100.84%
	12	11.78	98.17%	12.12	101.00%		5.0	5.06	101.21%
	20	20.16	100.80%	19.83	99.13%		15	14.83	98.91%
20	20	20.03	100.13%	20.00	100.01%	15	15	14.95	99.72%
	20	20.20	100.99%	20.09	100.46%		15	15.08	100.53%
	30	30.27	100.89%	29.43	98.09%		30	29.57	98.59%
30	30	30.40	101.33%	29.69	98.97%	30	30	29.45	98.18%
	30	30.13	100.45%	29.87	99.56%		30	29.69	98.99%
Accuracy(Mean)			100.39%		99.83%	Accuracy(Mean)			99.87%

Accuracy is further assessed by applying the standard addition technique to Azelast 0.05%® Eye Drops, where good recoveries are obtained revealing that there was no interference from excipients, Table 3.

Table 3. *Determination of AZH and BAC in their pharmaceutical formulation by the proposed methods and application of standard addition technique.*

Pharmaceutical formulation	Drug	Method	Found%[a]Mean±RSD	Standard addition		
		[D^0]λ=284 nm	100.70±0.80	Added µg/mL	Found µg/mL	Recovery %[b]
	AZH			10	9.96	99.64
				15	15.22	101.48
				20	19.99	99.97
				Mean±RSD		100.36±0.97
Azelast 0.05% Eye Drops AZH, 0.5 mg (claimed) BAC, 0.1mg (claimed)		[RD](262–208 nm)	100.42±0.30	10	9.95	99.50
				15	15.19	101.30
				20	20.19	100.96
				Mean±RSD		100.59±0.95
	BAC	[RS]λ=208 nm	99.73±0.63	10	9.93	99.37
				15	15.10	100.69
				20	20.21	101.06
				Mean±RSD		100.37±0.88

[a]Average of 6 determinations.
[b]Average of 3 determinations

3.4.6. Selectivity

The selectivity of the laboratory prepared mixtures containing both drugs in different ratios within the linearity range led to the gain of satisfactory results as shown in Table 4.

Table 4. *Determination of AZH and BAC in laboratory prepared mixture by applying the proposed methods.*

Ratio of AZH:BAC	Added (µg/mL) AZH	Added (µg/mL) BAC	AZH		BAC
			*Recovery %		
			[D^0]λ=284 nm	[RD](262–208 nm)	[RS]λ=208 nm
5:1	25.00	5.00	100.04	99.22	99.73
4:2	20.00	10.00	101.09	100.51	99.04
3:2	15.00	10.00	99.86	100.51	100.50
5:3	25.00	15.00	99.71	99.81	98.90
2:1	10.00	5.00	100.45	100.02	99.20
1:1	5.00	5.00	99.87	100.64	100.84
3:4	15.00	20.00	100.13	100.35	100.16
Mean±RSD			100.16±0.47	100.15±0.50	99.77±0.76

*Average of three determinations

The obtained results for the analysis of AZH and BAC by the suggested methods are statistically compared with the reported HPLC method using student's-t and F-ratio tests, showing that both methods are accurate and precise as presented in Table 5.

Table 5. *Statistical comparison between the results obtained by applying the proposed methods and the reported method for analysis of Azelastine hydrochloride and Benzalkonium* chloride *in their pharmaceutical formulation.*

Method	Reported Method[a] [25]		[D^0]λ=284 nm	[RD](262–208 nm)	[RS]λ=208 nm
	AZH	BAC	AZH	BAC	BAC
Mean	101.35	101.56	100.70	101.42	100.84
SD	1.49	1.39	0.80	1.10	1.46
Variance	2.22	1.95	0.65	1.21	2.14
N	6	6	6	6	6
t - test[b]	-	-	0.930	0.193	0.930
F - value[b]	-	-	1.49`	1.61	1.09

[a]RP-HPLC Method for Simultaneous Estimation of Fluticasone propionate, Azelastine Hydrochloride, Phenylethyl alcohol and Benzalkonium chloride using CN column (5µm, 25cm x 4.6 mm I.D.),50 mM of potassium dihydrogenorthophosphate: acetonitrile(55:45,v/v) as the mobile phase at a flow rate of 1.0 mL/min and UV detection at 215 nm.
[b]the values in parenthesis are corresponding to the theoretical values of t and F (p=0.05).

4. Conclusion

The proposed spectrophotometric methods for the determination of azelastine hydrochloride and benzalkonium chloride in combined ophthalmic solution is simple, precise, specific, accurate, less time consuming, low cost and rapid. Hence, this method is recommended for routine quality control analysis.

References

[1] British Pharmacopoeia, Stationary Office, Medicines and Healthcare Products Regulatory Agency, London, Vol. II, 2017.

[2] The Merck Index: An Encyclopedia of Chemicals, Drugs and Biological., S. Budavari (Ed.), Merck &Co., Inc., Whitehouse Station, NJ 15th Ed, 2006.

[3] S. C. Sweetman, Martindale: The Complete Drug Reference, 36th Ed., The Pharmaceutical Press, London, 2007.

[4] J. M. Horbal, J. A. Bernstein, "Azelastine HCl: A Review of the Old and New Formulations", Journal of Clinical Medicine Insights: Therapeutics, 2; 427-437, 2010.

[5] G. A. Gomez, A. M. Gonzalez, T. J. Garces, S. J. Costa, "Determination of benzalkonium chloride in aqueous ophthalmic preparations by high-performance liquid chromatography", Journal of Pharm Biomed Anal. 8(8-12); 871-6, 1990.

[6] European Pharmacopeia 8th edition, 2014.

[7] The United States Pharmacopoeia, 38th Revision, NF33, The United States Pharmacopoeia Convention Inc., June1, 2015.

[8] A. A. Gouda, R. ElSheikh, H. ElSaied, "Extractive Spectrophotometric Determination of Azelastine Hydrochloride in Pure Form and Pharmaceutical Formulations", Canadian Chemical Transactions, 3 (1); 29-41, 2015.

[9] T. Haraguchi, M. S. Nothenberg, "Volumetric and spectrophotometric assays of azelastine hydrochloride", Rev. Bras. Cienc. Farm.35; 127–131, 1999.

[10] N. N. Salama, S. A. Abdel-razeq, S. Abdel-Atty, N. El-Kosy, "Spectrophotometric determination and thermodynamic studies of the charge transfer complexes of azelastine-HCl", Bull. Fac. Pharm. Cairo Univ., 49; 13-18, 2011.

[11] R. T. Sane, S. G. Joshi, M. Francis, A. R. Khatri, P. S. Hijli, "Extractive colorimetric determination of Azelastine hydrochloride from its pharmaceutical preparations", Indian Drugs, 36; 317–320, 1999.

[12] M. R. Elghobashy, O. M. Badran, M. Y. Salem, K. M. Kelani, "Stability indicating spectrophotometric and chromatographic methods for the determination of azelastine hydrochloride in presence of its alkaline degradant", Analytical Chemistry: An Indian J., 14 (4), 2014.

[13] H. A. Merey, S. S. El-Mosallamy, N. Y. Hassan, B. A. El-Zeany, "Simultaneous determination of Fluticasone propionate and Azelastine hydrochloride in the presence of pharmaceutical dosage form additives", Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 160; 50-57, 2016.

[14] R. T. Sane, S. G. Joshi, M. Francis, A. R. Khatri, P. S. Hijli, "High-performance thin layer chromatographic determination of azelastine hydrochloride", J. Planar. Chromatogr. modern TLC, 12; 158–160, 1999.

[15] N. N. Salama, S. A. Abdel-Razeq, S. Abdel-Atty, N. El-Kosy, "Development and validation of densitometry TLC stability indicating method for quantitative determination of azelastine hydrochloride and emedastine difumarate in their drug products", British J. Pharm. Res., 4; 79-92, 2014.

[16] R. Dubey, S. Das, S. Roychowdhury, K. K. Pradhan, M. Ghosh, "Validated HPTLC Method for the Determination of Azelastine hydrochloride in Bulk Drug and Dosage Form", PHARMBIT, 27 (1); 9-11, 2013.

[17] E. Wyszomirska, K. Czerwi-Ska, E. Kublin, A. P. Mazurek, "Identification and determination of Ketotifen hydrogen fumarate, Azelastine hydrochloride, Dimetindene maleate and Promethazine hydrochloride by densitometric method", Acta Poloniae Pharmaceutica-DrugResearch, 70 (6); 951-959, 2013.

[18] B. Koppenhoefer, A. Jakob, X. F. Zhu, B. C. Lin, "Separation of enantiomers of drugs by capillary electrophoresis with permethyl-gama-cyclodextrin as chiral solvating agent", J. High. Resol. Chromatogr. 23; 413–429, 2000.

[19] N. Suzuki, Y. Ishihama, T. Kajima, N. Asakawa, "Quantitation of counterion of a water-insoluble drug by nonaqueous capillary electrophoresis within direct UVdetection", J. Chromatogr. A., 829, 411, 1998.

[20] Y. S. Park, S. H. Kim, Y. J. Kim, S. C. Yang, M. H. Lee, L. M. Shaw, "Determination of Azelastine in Human Plasmaby Validated Liquid Chromatography Coupled to Tandom Mass Spectrometry (LCESI/MS/MS) for the Clinical Studies", Int. J. Biomed. Sci., 6; 120–127, 2010.

[21] U. Heinemann, G. Blaschke, N. Knebel. "Simultaneous enantitio selective separation of azelastine and three of its metabolites for the investigation of the enantiomeric metabolism in rats. Liquid Chromatography ion spray tandem mass spectrometry and electrokinetic capillary chromatography", J. Chromatogr. B. Anal. Technol. Biomed. Life. Sci., 793; 389–404, 2003.

[22] T. Kajima, N. Asakawa, T. Hattori, T. Sato, Y. Miyake, "Optical resolution by high performance liquid chromatography, separation of enantiomeric azelastine hydrochloride by ionpair chromatography", YakugakuZasshi, 109; 570–573, 1989.

[23] P. Yoo-Sin, K. Shin-Hee, K. Young-Jae, Y. Seok-Chul, L. Min-Ho, M. S. Leslie, K. Ju-Seop, "Determination of Azelastine in Human Plasma by Validated Liquid Chromatography Coupled toTandom Mass Spectrometry (LC-ESI/MS/MS) for the Clinical Studies", Int. J. Biomed. Sci., 6; 120-127, 2010.

[24] T. Boovizhikannan, V. K. Palanirajan, "RP-HPLC determination of azelastine in pure and in ophthalmic formulation", Int. J. Pharm. Sci. Rev. Res, 17, 62, 2012.

[25] K. L. N. Rao, K. P. Reddy, K. S. Babu, K. S. Raju, K. V. Rao, J. V. Shaik, "Simultaneous Estimation of Fluticasone propionate, Azelastine Hydrochloride, Phenyl ethyl alcohol and Benzalkonium chloride by RP-HPLC Method in Nasal spray preparations", International Journal of Research in Pharmaceutical Sciences1 (4), 473-480, 2010.

[26] C. N. Langevin, J. Pivonka, J. K. Wichmann, N. Kucharczyk, R. D. Sofia, "High performance liquid chromatographic determination of azelastine and desmethyl azelastine in guinea pig plasma and lung tissue", Biomedical Chromatography. 7 (1): 7-11, 1993.

[27] K. K. Rahul, S. R. Baviskar, S. K. Mahajan, "Assay Method Development and Validation for Simultaneous Estimation of Mometasone Furoate and Azelastine Hydrochloride by RP-HPLC", Asian Journal of Biochemical and Pharmaceutical Research, 4 (4), 31-39, 2014.

[28] S. A. Abdel-razeq, M. M. Foaud, N. N. Salama, S. Abdel-Atty, N. El-Kosy, "Voltammetric determination of azelastine-HCl and emedastine difumarate in micellar solution at glassycarbon and carbon paste electrodes", Sensing in Electroanalysis. 6; 289-305, 2011.

[29] M. R. Elghobashy, O. M. Badran, M. Y. Salem, K. M. Kelani," Application of Membrane Selective Electrodes for the Determination of Azelastine Hydrochloride in the Presence of its Alkaline Degradant in Eye Drops and Plasma", Anal. Bioanal. Electrochem., 5; 325-340, 2013.

[30] S. A. Abdel-Razeq, N. N. Salama, S. Abdel-Atty, N. El-Kosy, "Thermo analytical study and purity determination of azelastine hydrochloride and emedastine difumarate", Pharm. Anal. Acta. 3 (8) 176-179, 2012; doi: 10.4172/2153-2435.1000176.

[31] J. J. Halvax, G. Wiese, J. A. Arp, J. M. Vermeer, W. P. VanBennekom, A. Bult, "Rapid determination of benzalkonium chloride in pharmaceutical preparations with flow injection liquid-liquid extraction", J Pharm Biomed Anal., 8 (3); 243-52., 1990.

[32] K. Kova´cs-Hadady, I. Fa´bia´n, "The determination of benzalkonium chloride in eye-drops by difference spectrophotometry", Journal of Pharmaceutical and Biomedical Analysis, 16; 733–740, 1998.

[33] J. E. Parkin, "The assay of benzalkonium chloride in pilocarpine, hypromellose and polyvinyl alcohol ophthalmic drops by second-order derivative ultraviolet spectrophotometry", J Pharm Biomed Anal., 11 (7): 609-11, 1993.

[34] A. Chyła, E. Zelazowska, "Using of thin-layer chromatography for identification and quantitative determination of benzalkonium chloride in eye drops", Acta Pol Pharm., 56 (6); 419-23, 1999.

[35] R. B. Taylor, S. Toasaksiri, R. G. Reid, "Capillary electrophoresis and liquid chromatography in the analysis of some quaternary ammonium salts used in lozenges as antibacterial agents", J Capillary Electrophor., 5 (1-2); 45-50, 1998.

[36] Y. H. Hou, C. Y. Wu, W. H. Ding, "Development and validation of acapillary zone electrophoresis method for the determination of benzalkonium chlorides in ophthalmic solutions", J Chromatogr A., 976 (1-2); 207-13,2002.

[37] M. Jimidar, I. Beyns, R. Rome, R. Peeters, G. Musch, "Determination of benzalkonium chloride in drug formulations by capillary electrophoresis (CE)", Biomed Chromatogr., 12 (3); 128-30, 1998.

[38] Z. R. Cybulski, "Determination of benzalkonium chloride by gas chromatography", J Pharm Sci., 73 (12): 1700-2, 1984.

[39] J. Dudkiewicz-Wilczynska, J. Tautt, I. Roman, "Application of the HPLC method for benzalkonium chloride determination in aerosol preparations", J Pharm Biomed Anal., 34 (5); 909-20, 2004.

[40] A. Gomez-Gomar, M. M. Gonzalez-Aubert, J. Garces-Torrents, J. Costa-Segarra, "Determination of benzalkonium chloride in aqueous ophthalmic preparations by high-performance liquid chromatography", J Pharm Biomed Anal., 8 (8-12);871-6,1990.

[41] A. A. Al-Fakhory, I. Al-Kalak, M. J. Al-khatib, "Chromatographic Determination of Total Benzalkonium Chloride [BAC] in Some of Ophthalmic Preparations by HPLC", Bull. Damascus Univ. for Basic Sciences. 30 (2), 155-171, 2014.

[42] F. Liu, K. P. Xiao, A. M. Rustum, "Determination of individual homologues and total content of benzalkonium chloride by reversed-phase high-performance liquid chromatography using a short butyl column", JAOAC Int., 92 (6): 1644-51, 2009.

[43] S. J. Prince, H. J. McLaury, L. V. Allen, P. McLaury, "Analysis of benzalkonium chloride and its homologs: HPLC versus HPCE", J Pharm Biomed Anal., 19 (6); 877-82, 1999.

[44] G. R. Takeoka, L. T. Dao, R. Y. Wong, L. A. Harden, "Identification of benzalkonium chloride in commercial grapefruit seedextracts", JAgric Food Chem., 53 (19); 7630-6, 2005.

[45] G. l. Santoni, A. Medica, P. Gratteri, S. Furlanetto, SPinzauti, "High-performance liquid chromatographic determination of benzalkonium and naphazoline or tetrahydrozoline in nasal and ophthalmic solutions", Farmaco.1994, 40 (11); 751-4, 1994.

[46] K. Kümmerer, A. Eitel, U. Braun, P. Hubner, F. Daschner, G. Mascart, M. Milandri, F. Reinthaler, J. Verhoef, "Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection", J Chromatogr A., 774 (1-2); 281-6, 1997.

[47] G. Ambrus, L. T. Takahashi, P. A. Marty, "Direct determination of benzalkonium chloride in ophthalmic systems by reversed-phase high-performance liquid chromatography", J Pharm Sci., 76 (2); 174-6, 1987.

[48] J. r. ElrodL, T. G. Golich, J. A. Morley, "Determination of benzalkonium chloride in eye care products by high-performance liquid chromatography and solid-phase extraction or on-line column switching", J Chromatogr., 625 (2); 362-7, 1992.

[49] T. Y. Fan, G. M. Wall, "Determination of benzalkonium chloride in ophthalmic solutions containing tyloxapol by solid-phase extraction and reversed-phase high-performance liquid chromatography", J Pharm Sci., 82 (11); 1172-4,1993.

[50] R. Mallik, S. Raman, X. Liang, A. W. Grobin, D. Choudhury, "Development and validation of a rapid ultra-high performance liquid chromatography method for the assay of benzalkonium chloride using a quality-by-design approach", J Chromatogr A.,1413; 22-32, 2015.

[51] D. F. Marsh, L. T. Takahashi, "Determination of benzalkonium chloride in the presence of interfering alkaloids and polymeric substrates by reverse-phase high-performance liquid chromatography", J Pharm Sci., 72 (5); 521-5, 1983.

[52] L. P. Labranche, S. N. Dumont, S. Levesque, A. Carrier, "Rapid determination of total benzalkonium chloride content in ophthalmic formulation", J Pharm Biomed Anal., 43 (3); 989-93, 2006.

[53] R. C. Meyer, "Determination of benzalkonium chloride by reversed-phase high-pressure liquid chromatography", J Pharm Sci., 69 (10): 1148-50, 1980.

[54] P. Rojsitthisak, W. Wichitnithad, O. Pipitharome, K. Sanphanya, P. Thanawattanawanich, "Simple HPLC determination of benzalkonium chloride in ophthalmic formulations containing antazoline and tetrahydrozoline", PDA J Pharm Sci Technol., 59 (5); 332-7, 2005.

[55] J. Zakrajšek, V. Stojic, S. Bohanec, U. Urleb, "Quality by design based optimization of a high performance liquid chromatography method for assay determination of low concentration preservatives in complex nasal formulations", Acta Chim Slov., 62 (1); 72-82, 2015.

[56] E. Martínez-Carballo, C. González-Barreiro, A. Sitka, N. Kreuzinger, S. Scharf, O. Gans. "Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. PartII. Application to sediment and sludge samples in Austria"|, Environ Pollut., 146 (2); 543-7, 2007.

[57] H. AlAani, Y. AlNukkary. "Determination of Benzalkonium Chloride in Ophthalmic Solutions by Stability-Indicating HPLC Method: Application to a Stability Study". Journal of Applied Pharmaceutical Science, 6 (05); 080-089, 2016.

[58] M. E. M. Hassouna, M. M. Abdelrahman, M. A. Mohamed. "Simultaneous Determination of Azelastine hydrochloride and Benzalkonium chloride by RP-HPLC Method in their Ophthalmic Solution". J Forensic Sci & Criminal Inves., 1 (3); 555565-75, 2017.

[59] ICH, Q2(R1)Validation of Analytical Procedures: Text and Methodology. ICH Harmonized Tripartite Guideline, 2005.