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Determination of Benzalkonium Chloride in Ophthalmic Solutions by Stability-Indicating HPLC Method: Application to a Stability Study

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ABSTRACT

A simple HPLC method for determination of Benzalkonium chloride in various ophthalmic solutions was developed. The chromatographic analysis was achieved using CN column (250 mm, 4.6 mm i.d., 5 µm) with isocratic mobile phase consisting of acetonitrile - phosphate buffer (pH 5.5; 0.05 M) (70:30, v/v) at a flow rate of 1 mL/minute. The column temperature was maintained at 25°C and the detection wavelength was 210 nm. The proposed HPLC method was successfully validated according to the ICH guideline and proved to be stability-indicating. This method was applied to quantify Benzalkonium chloride during in-use stability study of two ophthalmic solutions. Antimicrobial effectiveness of Benzalkonium chloride in these solutions was also evaluated. The developed method is suitable for the routine analysis of Benzalkonium chloride in many ophthalmic solutions as well as for the stability studies.

INTRODUCTION

Ophthalmic preparations are sterile aqueous or oily solutions or suspensions of one or more active materials. These products are normally packed in suitable multi-dose containers that allow the instillation of successive drops of the preparation (Semwal *et al.,* 2014). Microbial contamination or proliferation during storage and use of ophthalmic preparations may lead to product spoilage or may cause serious ocular infections (Semwal *et al.,* 2014; BP, 2013). Protection of these multi-dose products is usually achieved by using of suitable preservatives (Semwal *et al.,* 2014). Benzalkonium chloride (BAC), a mixture of alkylbenzyldimethylammonium chlorides [Fig. 1] (USP, 2012), is the most commonly preservative used in various dosage forms including ophthalmic preparations (Liu *et al.,* 2009). It was first used in the 1940s; and since then, BAC has been used in nearly all classes of ophthalmic solutions, from antiglaucoma products to OTC artificial tear solutions (Freeman and Kahook, 2009). The general concentrations range of BAC used in pharmaceutical preparations is 0.002% – 0.02%, but it could be up to 0.2% in some cases, depending on different factors in ophthalmic formulations (Liu *et al.,* 2009).

In-use stability is a stability study applied for multi-dose products to ensure that the product preserves its quality parameters after opening during use period. The physical, chemical and microbial properties of the product susceptible to change during storage should be determined over the period of the proposed in-use shelf-life; and for liquids, preservatives, per content and effectiveness, need to be evaluated in this study (WHO, 2009).

A number of analytical methods have been reported for the estimation of BAC in various products including ophthalmic preparations (Agarwal *et al.,* 2013; Al-Fakhory *et al.,* 2014; Chiapetta *et al.,* 2011; Dudkiewicz-Wilczyńska *et al.,* 2004; Gaber *et al.,* 2012; Jovovic´ *et al.,* 2012; Kapakayala *et al.,* 2013; Kostić *et al.,* 2012; Mehta *et al.,* 2010; Rao *et al.,* 2013; Santos *et al.,* 2010; Shaikh and Patil, 2013; Shen *et al.,* 2009; Trivedi and Patel, 2010; Trivedi *et al.,* 2013). However, the described methods were

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limited to a number of ophthalmic preparations, and some of them determined the BAC as total not as homologs. Therefore, the aim of this work was to develop a simple stability-indicating HPLC method to determine BAC as homologs, and to prove its applicability in common ophthalmic solutions that contain one or more of the following active ingredients: Brimonidine tartrate, Chloramphenicol, Dexamethasone sodium phosphate, Dorzolamide hydrochloride, Ketorolac tromethamine, Latanoprost, Naphazoline hydrochloride, Olopatadine hydrochloride, Pheniramine maleate, Timolol maleate, and Tetrahydrozoline hydrochloride.

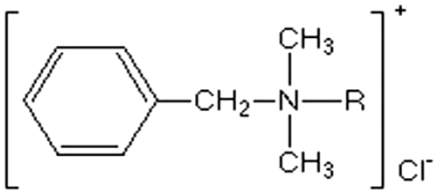


Fig. 1: Chemical structure of Benzalkonium chloride (BAC) in which R represents a mixture of alkyls, including all or some of the group beginning with n-C8H17 and extending through higher homologs, with n-C12H25, n-C14H29, and n-C16H33 composing the major portion

As application of the proposed method, it was used for BAC estimation in various marketed ophthalmic solutions and during in-use stability study.

MATERIALS AND METHODS

Chemicals and solutions

Benzalkonium chloride for system suitability reference standard was purchased from European Pharmacopoeia (EDQM), Batch No. 3.0. Benzalkonium chloride reference standard was purchased from U.S. Pharmacopoeia, Lot L1I130. Benzalkonium chloride 50% solution was purchased from Merck, Germany. All ophthalmic solutions, active ingredients, and excipients were kindly supplied by DIAMOND PHARMA, Syria. Acetonitrile used was of HPLC grade. All other reagents used were of analytical grade.

Chromatographic conditions

Analysis was performed with a HPLC (LaChrom ELITE, VWR-Hitachi, Germany, equipped with L-2130 pump, L-2200 auto sampler, L-2300 column oven, and UV photo diode array detector L-2455). The out-put signal was monitored and processed using EZ Chrom ELITE software**.**

A Macherey-Nagel Nucleodur 100-5 CN column with dimensions of 250 mm × 4.6 mm, 5 µm was used. A Thermo CPS Hypersil column with same dimensions was used in robustness study.

The isocratic mobile phase comprised of acetonitrile - potassium dihydrogen phosphate buffer (pH 5.5; 0.05 M) (70:30, v/v). The mobile phase was filtered through 0.45 µm membrane filter, degassed in ultrasonic bath and pumped from the respective solvent reservoir to the column at a flow rate of 1 mL/minute. All analysis was done at 25°C and the detection wavelength was 210 nm. The injection volume was 50 µL.

Method validation

The proposed HPLC method was validated according to ICH guideline (ICH, 2005), with the aspect of system suitability, specificity, linearity, precision, accuracy, robustness, carryover and filter validation.

Forced degradation studies

Forced degradation studies were conducted to prove the stability-indicating property of the developed method.

Stock solution

Benzalkonium chloride stock solution having a concentration of 5 mg/mL was prepared in mobile phase and spiked with placebo.

Acidic degradation study

1 mL of stock solution was transferred into a 50 mL volumetric flask containing 20 mL of mobile phase. 1 mL of 1 M HCl was added to the volumetric flask, and then the flask was kept at 70°C for about 1 hour in water bath. Then the solution was allowed to attend ambient temperature, neutralized by 1 M NaOH, and the volume was made up to the mark with mobile phase.

Alkaline degradation study

1 mL of stock solution was transferred into a 50 mL volumetric flask containing 20 mL of mobile phase. 5 mL of 1 M NaOH was added to the volumetric flask, and then the flask was kept at 70°C for about 1 hour in water bath. Then the solution was allowed to attend ambient temperature, neutralized by 1 M HCl, and the volume was made up to the mark with mobile phase.

Oxidative degradation study

1 mL of stock solution was transferred into a 50 mL volumetric flask containing 20 mL of mobile phase. 1 mL of 3% H_2O_2 was added to the volumetric flask, and then the flask was kept at 70°C for about 1 hour in water bath. Then the solution was allowed to attend ambient temperature and the volume was made up to the mark with the mobile phase.

Thermal degradation study

1 mL of stock solution was transferred into a 50 mL volumetric flask containing 20 mL of mobile phase. The volumetric flask was kept at 70°C for 4 hours in water bath. Then the solution was allowed to attend ambient temperature and the volume was made up to the mark with mobile phase.

Photolytic degradation study

1 mL of stock solution was transferred into a 50 mL volumetric flask containing 20 mL of mobile phase. The solution was subjected to both of the cool white fluorescent and near ultraviolet lamp with a maximum energy emission at 365 nm for 4

hours. Then the solution was allowed to attend ambient temperature and the volume was made up to the mark with mobile phase.

All treated solutions were filtered with a 0.45 µm nylon syringe filter and injected in stabilized chromatographic conditions.

Application of the developed method

Analysis of Benzalkonium chloride in ophthalmic solutions

The developed method was applied to determine BAC content in the following ophthalmic preparations: Latanoprost; Latanoprost & Timolol maleate; Brimonidine tartrate; Brimonidine tartrate & Timolol maleate; Dexamethasone sodium phosphate, Chloramphenicol & Tetrahydrozoline hydrochloride; Ketorolac tromethamine; Olopatadine hydrochloride; Dorzolamide hydrochloride & Timolol maleate; Pheniramine maleate & Naphazoline hydrochloride; and Tetrahydrozoline hydrochloride.

5 mL of each of these solutions was diluted to 10 mL with mobile phase, filtered using a 0.45 µm nylon syringe filter, and injected in stabilized chromatographic conditions.

Benzalkonium chloride percentage was calculated in comparison with a standard solution having a corresponding concentration using the following formula:

$$\text{BAC \%} = (r_U/r_S) \times (C_S/C_U) \times P$$

In which:

r_U and r_S are sum of the peak areas for all BAC homologs obtained from the sample solution and the standard solution, respectively.

C_S is the concentration, in µg per mL, of BAC in the standard solution.

C_U is the nominal concentration, in µg per mL, of BAC in the sample solution.

P is the potency of standard (as percentage).

In-use stability study

In-use stability study was applied for Latanoprost & Timolol maleate, and Dorzolamide hydrochloride & Timolol maleate ophthalmic solutions. The study was performed at 30°C ± 2°C/65% RH ± 5% RH.

Products, at the end of their shelf-life, were treated in a manner simulates the use in practice. At the end of proposed in-use period, the remaining amounts of samples were tested.

Part of the applied tests was determining the change in BAC content during in-use shelf-life; therefore BAC was assayed at the initial (T_0) and after 30 days of opening (T_{30}), using the developed HPLC method.

This stability study included also evaluating of BAC antimicrobial effectiveness, which carried out as prescribed in US Pharmacopoeia (USP, 2012).

RESULTS AND DISCUSSION

Method validation

Suitability test was performed to ascertain the effectiveness of the operating chromatographic system, by evaluating specified parameters from five replicate injections of standard solution (100 µg/mL of BAC in mobile phase). The results for each of BAC homologs were within the acceptable limits as per FDA guideline (FDA, 1994), as shown in Table 1.

Table 1: System suitability test results.

Parameter	Standard value	BAC homolog 1	BAC homolog 2	BAC homolog 3	BAC homolog 4
RSD% of area	≤ 1% for n=5	0.13	0.39	0.95	0.38
RSD% of retention time	≤ 1% for n=5	0.35	0.36	0.41	0.44
Tailing factor (T)	≤ 2	1.36	1.24	1.19	1.22
Capacity factor (k')	> 2	2.60	-	-	-
Resolution (R)	> 2	-	2.26	2.33	2.39
Theoretical plates (N)	> 2000	6587	7421	7875	7791

Specificity of the method was assessed by comparing the chromatograms obtained from reference standards [Fig. 2 - 3], Benzalkonium chloride 50% solution [Fig. 4], and placebo solutions [Fig. 5] that were synthetic solutions consisted of all components of the studied ophthalmic preparations other than Benzalkonium chloride. The comparison showed no interfering peaks at the retention times of BAC homologs in the placebo chromatograms that indicates the specificity of the method. Specificity was further studied by conducting the force degradation studies.

The method was linear over the range of 12.5 µg/mL to 400 µg/mL, as the calibration curve - plotted over 6 different concentrations - had a correlation coefficient (R^2) value of 0.999937. The calibration curve is shown in [Fig. 6].

The method precision was assessed through repeatability (intra-day) and intermediate precision. Six determinations at concentration of 100 µg/mL were performed on the same day and under the same conditions for repeatability (intra-day). The intermediate precision was carried out at three concentration levels (25, 100, and 200 µg/mL), three replicates for each level by three different analysts. RSD% values less than 2% indicate the precision of the method. Recovery tests were done to assess the accuracy of the described method, which carried out by the spiked – placebo recovery method at three concentration levels (25, 100, and 200 µg/mL), in three replicates for each level. The recoveries obtained confirmed that the proposed method was accurate. The results of accuracy and precision studies of the proposed method are tabulated in Table 2.

The robustness of the method was evaluated to assure its reliability during normal usage, by making small changes in some method parameters including mobile phase pH (± 0.1), flow rate (± 0.1 mL/minute), and wavelength (± 3 nm), in addition to use another column from different supplier (Thermo CPS Hypersil column); System suitability parameters were measured from five replicate injections of standard solution under each condition, and found to be within the acceptable limits indicating that method was robust.

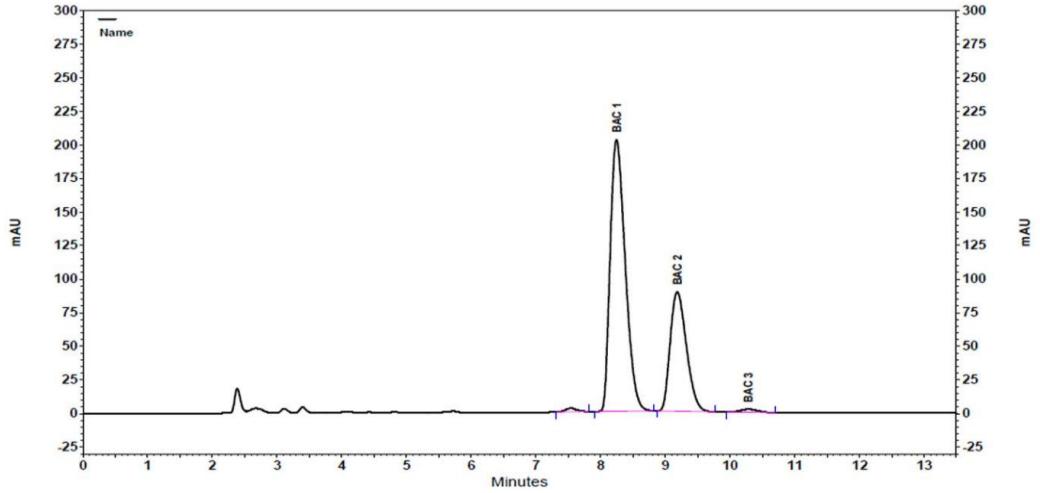


Fig. 2: Representative chromatogram of Benzalkonium chloride European Pharmacopoeia reference standard (80 µg/mL of BAC).

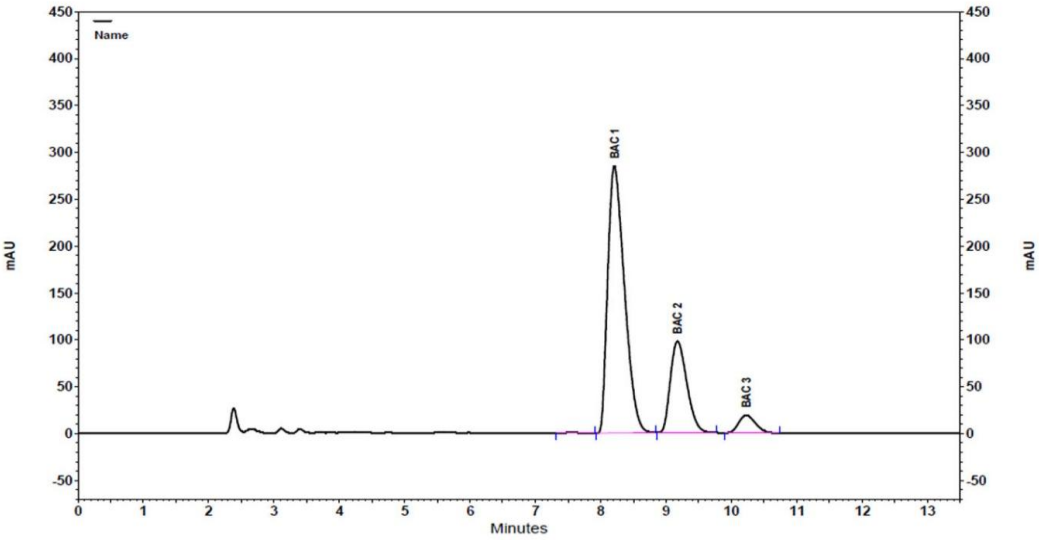


Fig. 3: Representative chromatogram of Benzalkonium chloride U.S. Pharmacopoeia reference standard (100 µg/mL of BAC).

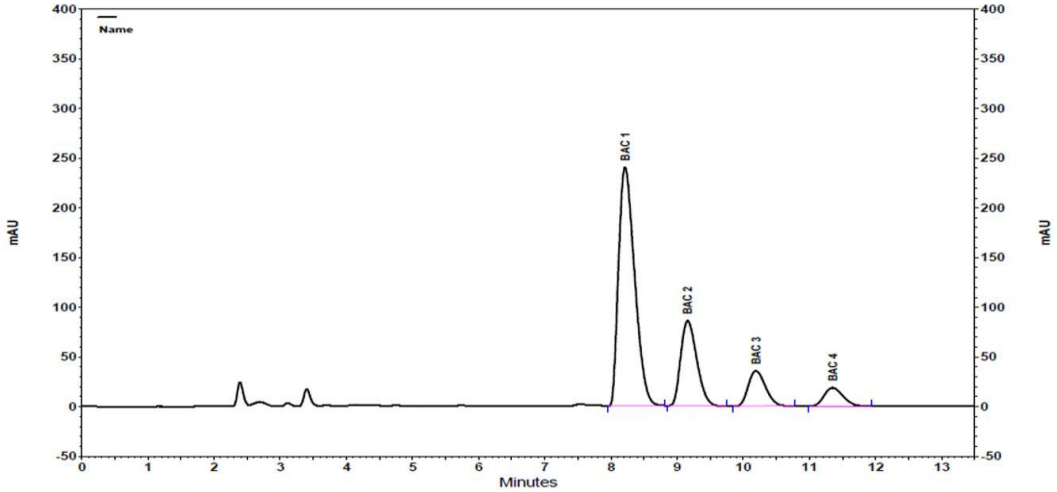
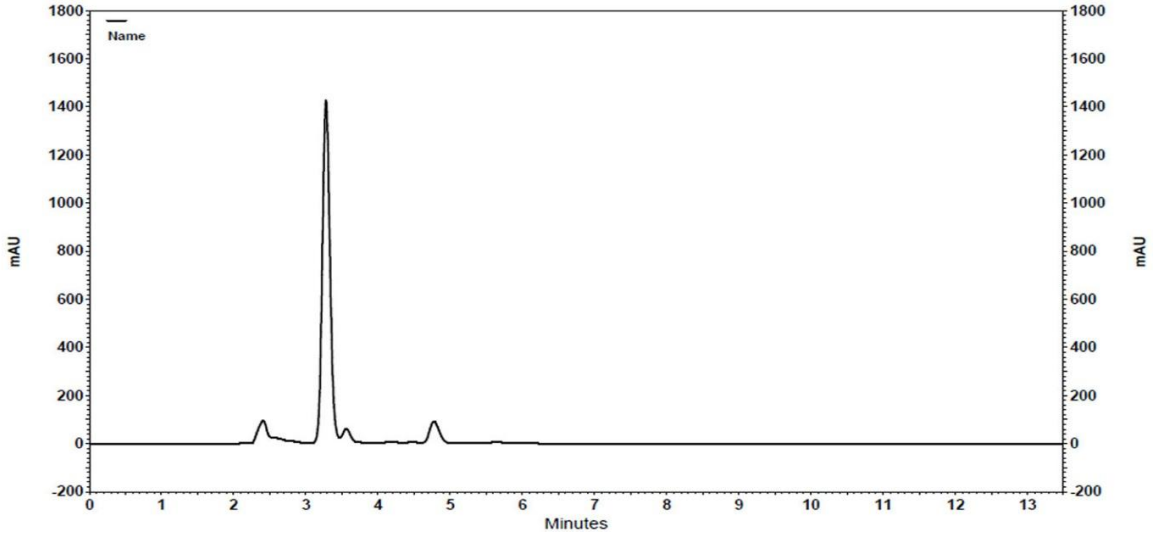
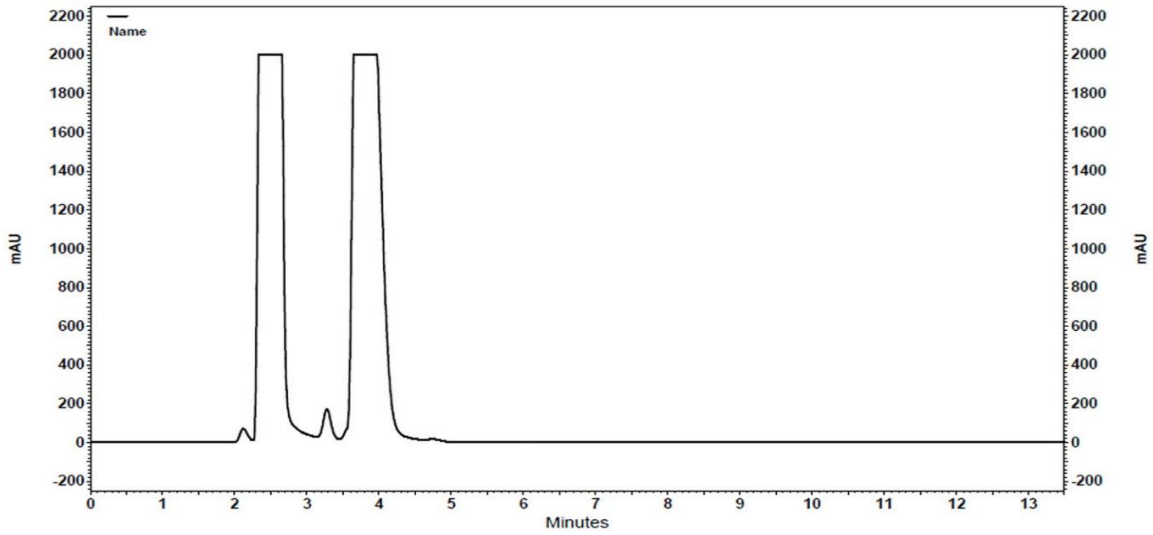


Fig. 4: Representative chromatogram of Benzalkonium chloride 50% solution (100 µg/mL of BAC).



A



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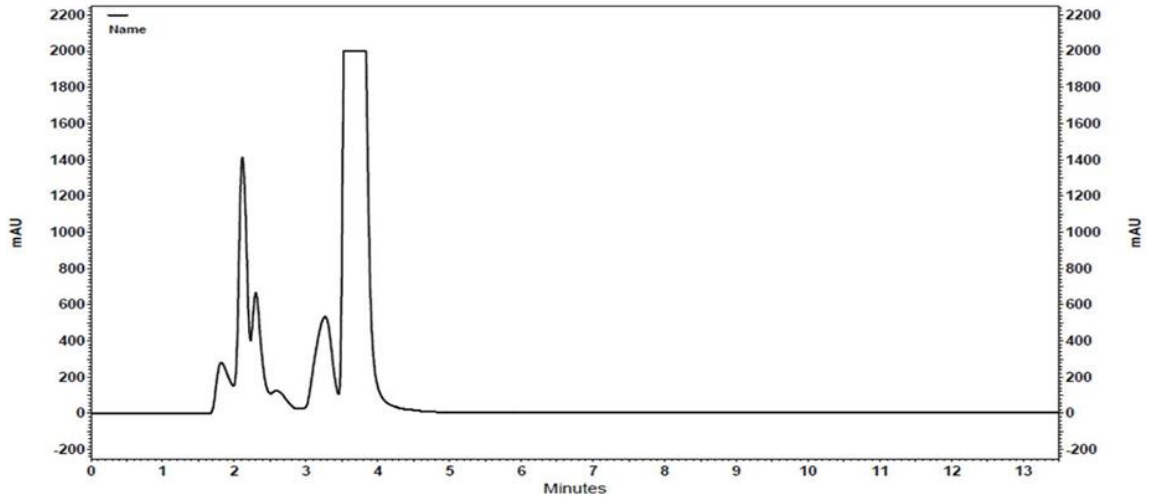

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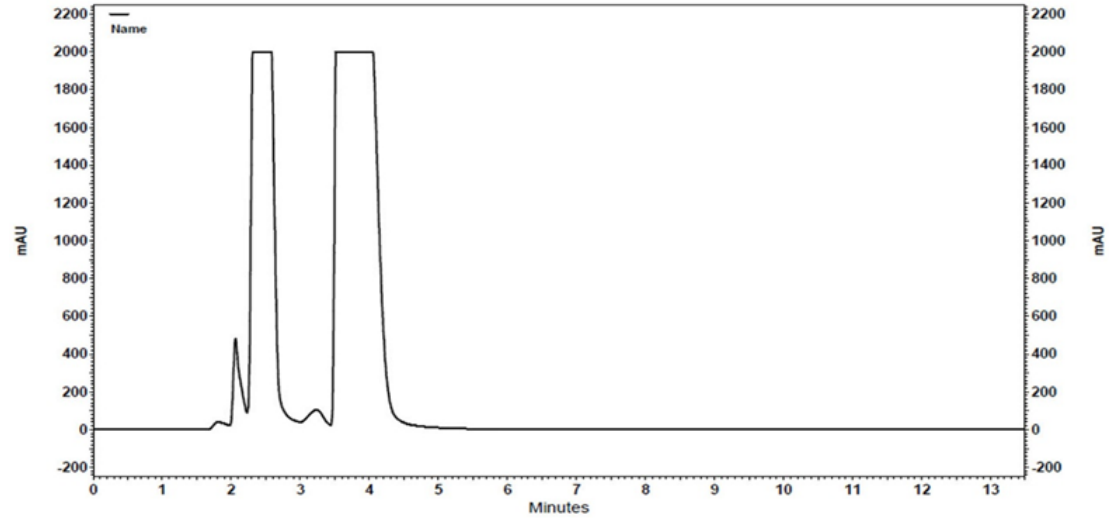

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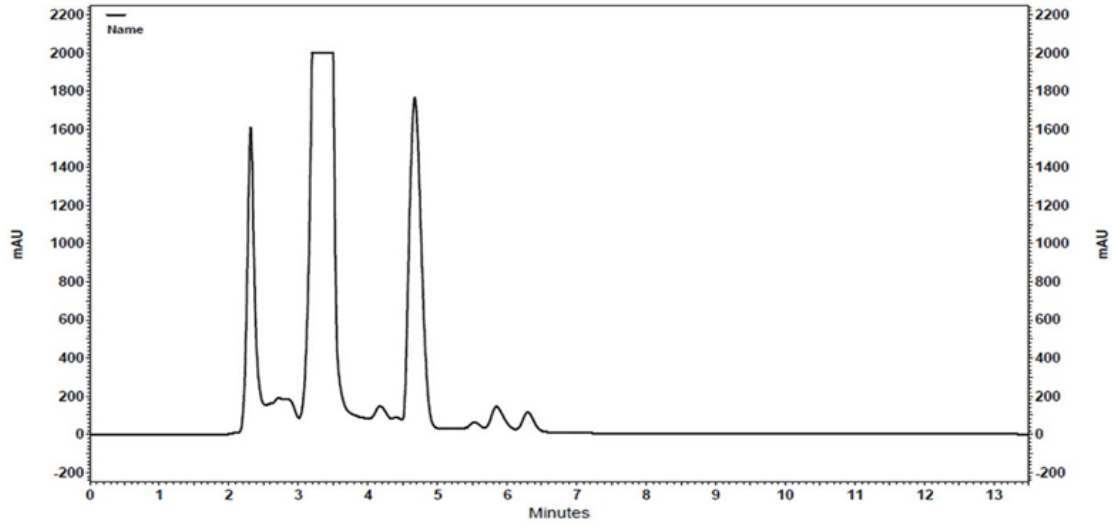

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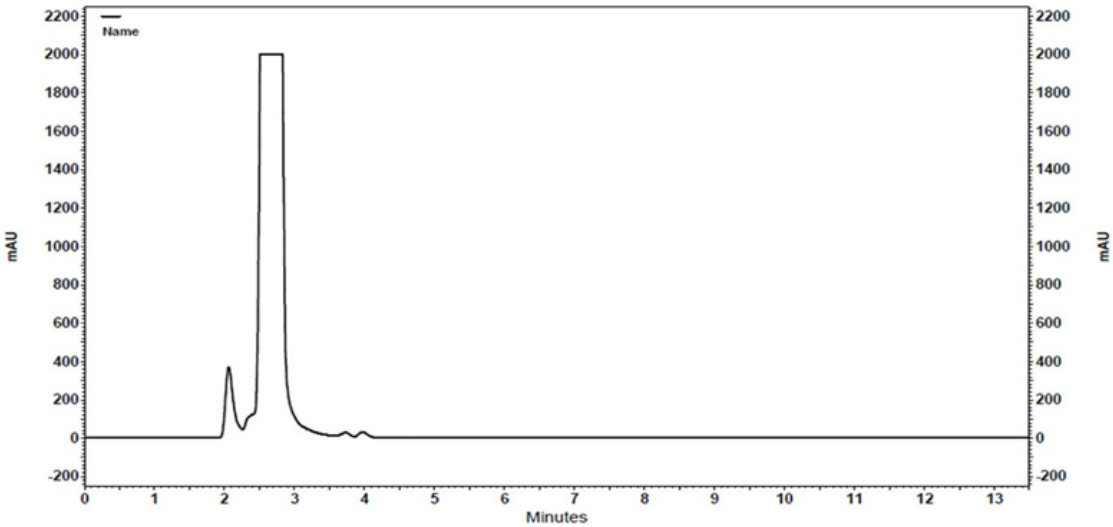

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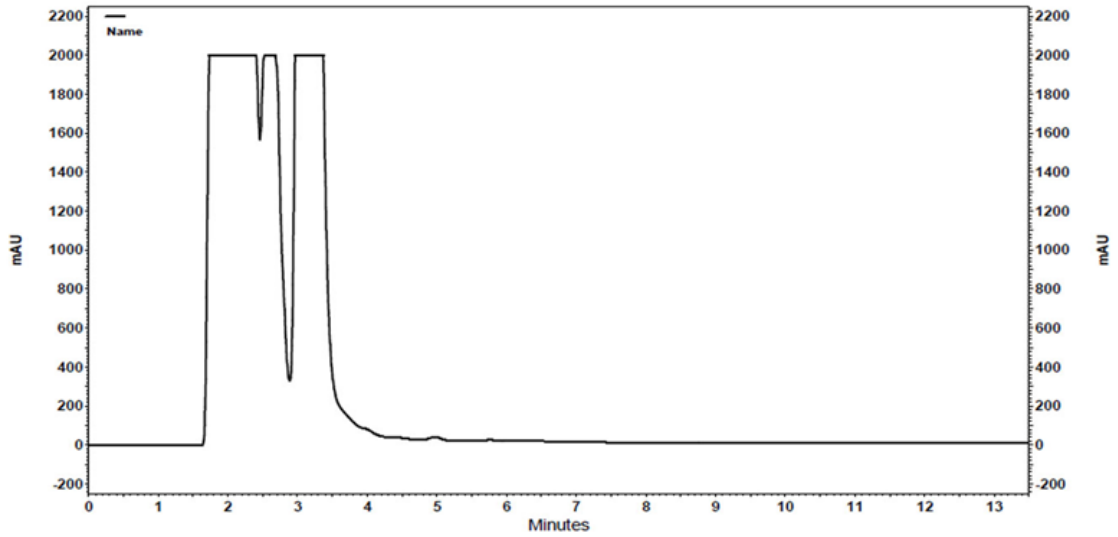

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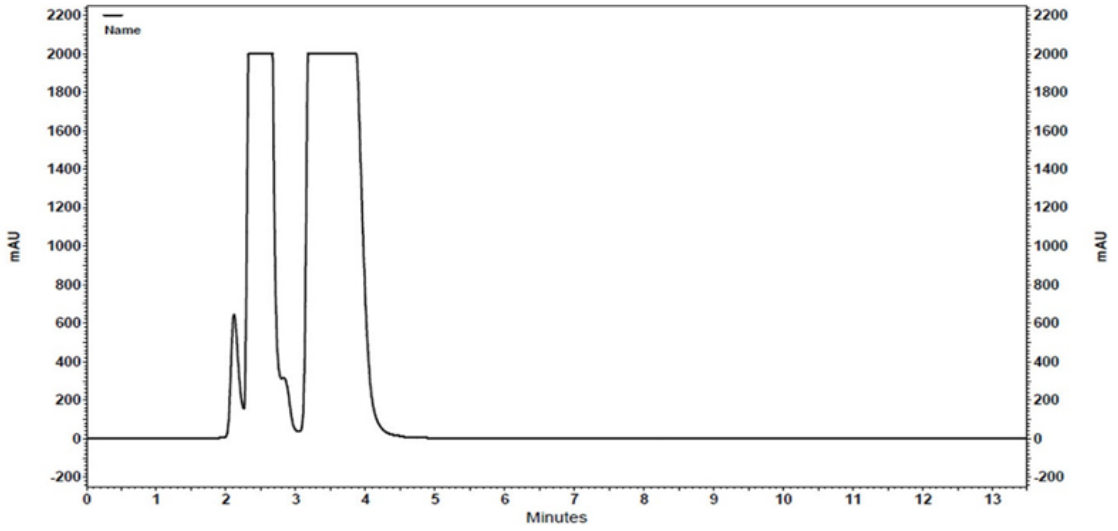

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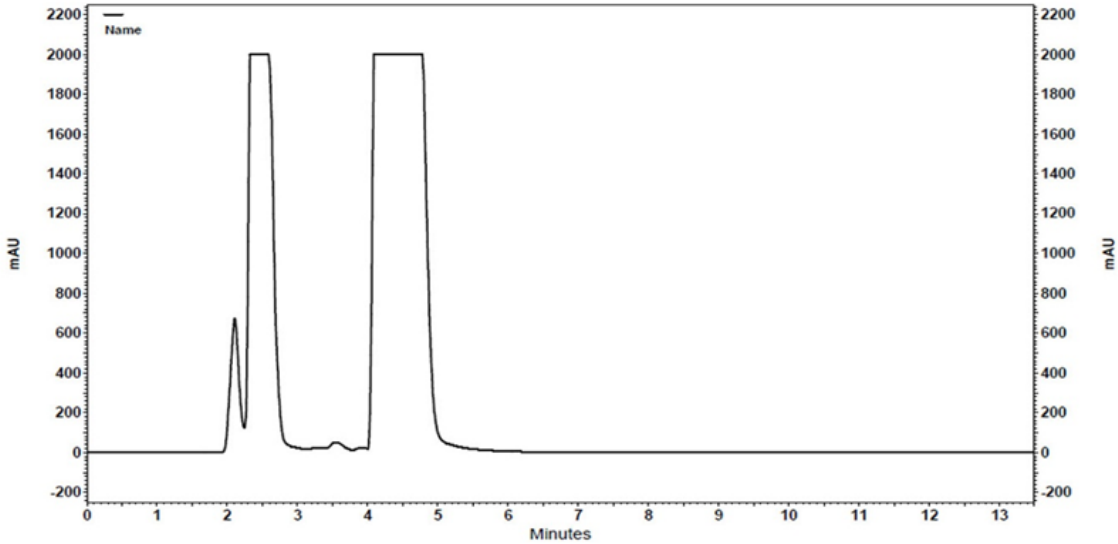

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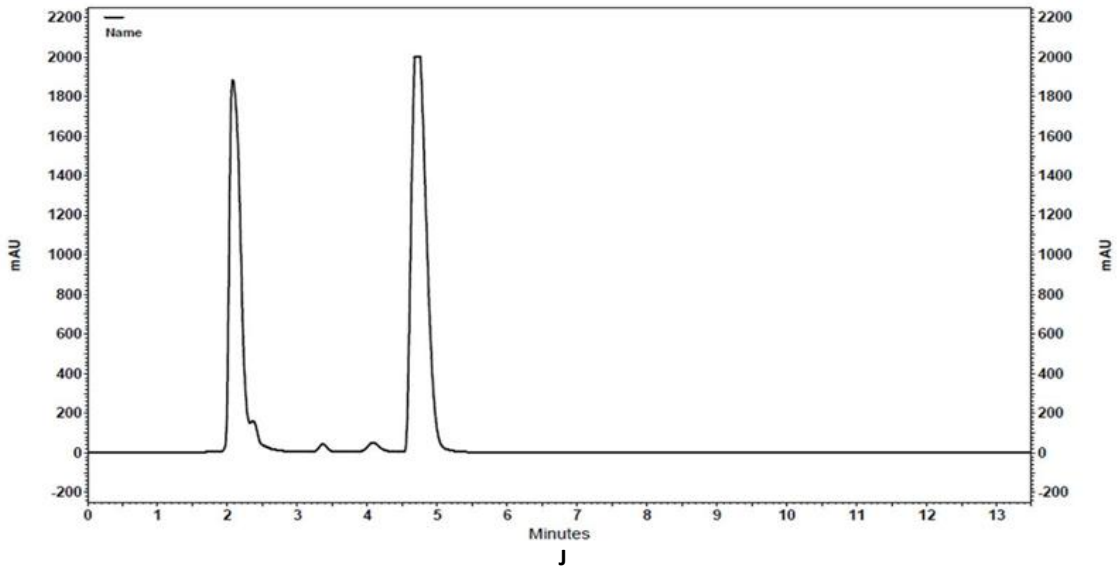

Fig. 5: Representative chromatograms of placebo solutions of the following ophthalmic solutions:
(A) Latanoprost; (B) Latanoprost & Timolol maleate; (C) Brimonidine tartrate; (D) Brimonidine tartrate & Timolol maleate; (E) Dexamethasone sodium phosphate, Chloramphenicol & Tetrahydrozoline hydrochloride; (F) Ketorolac tromethamine; (G) Olopatadine hydrochloride; (H) Dorzolamide hydrochloride & Timolol maleate; (I) Pheniramine maleate & Naphazoline hydrochloride; and (J) Tetrahydrozoline hydrochloride

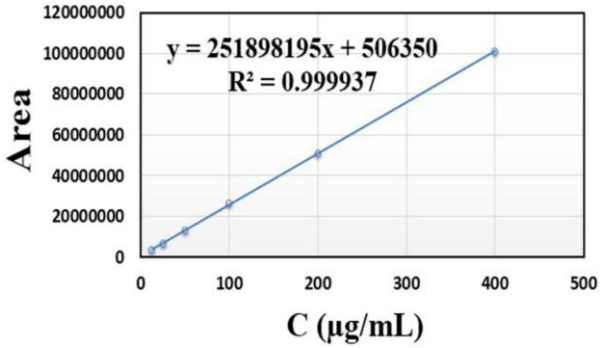

Fig. 6: Calibration curve of Benzalkonium chloride.

Table 2: Results of accuracy and precision studies.

Accuracy (Recovery %)			Precision (RSD%)			
			Repeatability (Intra-day)	Intermediate precision		
25 µg/mL	**100 µg/mL**	**200 µg/mL**	**100 µg/mL**	**25 µg/mL**	**100 µg/mL**	**200 µg/mL**
101.41% ± 0.41%	101.73% ± 0.71%	100.40% ± 0.49%	0.48%	0.72%	0.85%	0.40%

Carryover study

Carryover was evaluated by injecting mobile phase after solution with high concentration of BAC (400 µg/mL). Carryover was expressed as total BAC peaks area ratio of the mobile phase against that form BAC solution. Carryover percentage was found to be 0.48%, which is within the acceptance limit (≤ 1%) (Kassaye and Genete, 2013).

Filter validation

Adsorption of the BAC onto the 0.45 µm nylon syringe filter, used to prepare solutions for injection in the chromatographic system, was evaluated by analyzing unfiltered and filtered artificially prepared sample solutions, then comparing the results. Recovery percentage of BAC (filtered to unfiltered) was 100.59% ± 0.35%.

Forced degradation studies

After conducting the degradation conditions, BAC content was determined in the treated solutions. The results are presented in Table3. In the resulted chromatograms, no interference was observed between BAC homologs and the degradation products. The peak purity spectrum of BAC homologs was recorded using UV photo diode array detector. Peak purity results were greater than 0.99 indicating that the peaks were homogeneous in all stress conditions tested, which confirm the specificity and the stability-indicating property of the developed method.

Table 3: Results of forced degradation studies.

Stress condition	BAC Assay %
Acidic degradation	98.06% ± 0.45%
Alkaline degradation	94.62% ± 0.86%
Oxidative degradation	98.76% ± 0.54%
Thermal degradation	99.21% ± 0.52%
Photolytic degradation	99.28% ± 0.62%

Table 4: Results of Benzalkonium chloride content in ophthalmic solutions.

Ophthalmic solution	Labeled Content (mg/mL)	BAC content %
Latanoprost	0.2	104.09% ± 0.29%
Latanoprost & Timolol maleate	0.2	103.14% ± 0.72%
Brimonidine tartrate	0.05	104.88% ± 0.31%
Brimonidine tartrate & Timolol maleate	0.05	101.94% ± 0.68%
Dexamethasone sodium phosphate, Chloramphenicol & Tetrahydrozoline hydrochloride	0.1	100.83% ± 0.47%
Ketorolac tromethamine	0.1	105.00% ± 0.26%
Olopatadine hydrochloride	0.1	98.45% ± 0.22%
Dorzolamide hydrochloride & Timolol maleate	0.07	102.98% ± 0.34%
Pheniramine maleate & Naphazoline hydrochloride	0.1	101.28% ± 0.39%
Tetrahydrozoline hydrochloride	0.1	98.36% ± 0.83%

Table 5: Results of Benzalkonium chloride assay during in-use stability study.

Ophthalmic solution	BAC content %	
	T_0	T_{30}
Latanoprost & Timolol maleate	103.65% ± 0.40%	103.81% ± 0.63%
Dorzolamide hydrochloride & Timolol maleate	102.46% ± 0.54%	101.98% ± 0.50%

Table 6: Results of antimicrobial effectiveness test during in-use stability study.

	Acceptance criteria		Latanoprost & Timolol maleate solution		Dorzolamide hydrochloride & Timolol maleate solution	
	Bacteria	Yeast and Molds	Bacteria	Yeast and Molds	Bacteria	Yeast and Molds
7th day	NLT 1.0 log reduction	No increase	1.0 log reduction	No increase	1.0 log reduction	No increase
14th day	NLT 3.0 log reduction	No increase	3.9 log reduction	No increase	3.8 log reduction	No increase
28th day	No increase	No increase	No increase	No increase	No increase	No increase

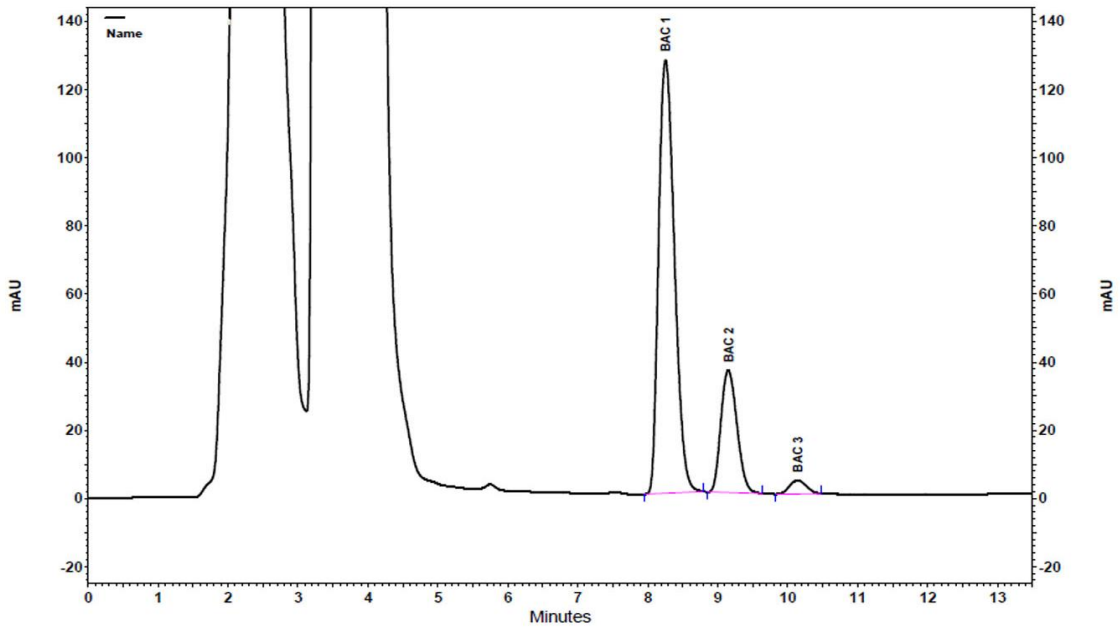

Fig. 7: Representative chromatogram of Dorzolamide hydrochloride & Timolol maleate ophthalmic solution (35 µg/mL of BAC)

Application of the developed method

Analysis of Benzalkonium chloride in ophthalmic solutions

The Benzalkonium chloride content in the tested preparations is presented in Table 4. The chromatogram resulted from analysis of Dorzolamide hydrochloride & Timolol maleate ophthalmic solution is represented in [Fig. 7], as an example.

In-use stability study

As per the results in Table 5 and 6, there was no significant change in BAC content during in-use period, and both of studied ophthalmic solutions passed the test for antimicrobial effectiveness according to US Pharmacopoeia (USP, 2012).

CONCLUSION

This study presents a simple validated HPLC method for estimation of BAC in a variety of ophthalmic preparations. The developed method is specific, rapid, robust, precise and accurate. The results of forced degradation studies imply that the developed method is stability-indicating. Developed method can be used as quality control tool for routine quantitative analysis of BAC and stability studies.

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