



Research Article

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An Analytical Study for Development and Validation of Method for Estimation of Total Benzalkonium chloride Content as a Preservative in Azelastine hydrochloride Pharmaceutical Ophthalmic Formulation by Reverse Phase Liquid Chromatography Approach

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ABSTRACT

A novel, sensitive, specific, accurate and reproducible reverse phase high performance liquid chromatographic analytical method (RP-HPLC) was developed and validated for estimation of total benzalkonium chloride (BKC) content as a preservative in azelastine hydrochloride pharmaceutical ophthalmic formulation. The reversed phase HPLC method was used with C18, cosmosil (250 mm × 4.6mm i.d. × 5µm) column. The mobile phase was used as a combination of acetonitrile and buffer (adjusted to pH 5.0 with 5N NaOH) in the ratio of 45:55 % v/v, mobile phase was pumped at a constant flow rate of 1.5 ml per minute. The quantification of benzalkonium chloride was carried out with UV detection at 210 nm and column oven temperature was 25°C. By using these chromatographic conditions of method, four homologues of benzalkonium chloride were separated without any interference of any drug product components. The obtained results were found linear in concentration range 40µg/ml to 60µg/ml (50µg/ml ± 20%), correlation coefficient of calibration curve of regression data was 0.999 value. Recovery was found to be 99.0%, 100.4% and 101.1% at ± 30% of theoretical target concentration. The %RSD for method precision, instrument precision were found 0.26% and 0.10% respectively. The method has been statistically validated as per international council for harmonization guideline Q2R1 and found within the acceptance criteria. So that the method was found specific, linear, precise and accurate for quantification of total benzalkonium chloride content as a preservative in azelastine hydrochloride pharmaceutical ophthalmic formulation.

Keywords: Benzalkonium chloride, HPLC method, benzalkonium chloride, preservative, ophthalmic solution.

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INTRODUCTION

Benzalkonium chloride is one of the most commonly used preservative, normally found in ophthalmic drug products and nasal formulations. [1] Benzalkonium chloride is one of a quaternary ammonium compound, a combined mixture of alkylbenzyltrimethylammonium chlorides containing the chemical formula $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R is an alkyl group changeable from C_8H_{17} to $C_{18}H_{37}$. [2] With a property of antimicrobial agent, benzalkonium chloride can be safely used in pharmaceuticals at lower concentrations from 0.002% to 0.02% but it can vary up to 0.2% in ophthalmic formulations depending on various factors. [3] Higher concentrations of benzalkonium chloride may cause damage to the corneal endothelium and found to be harmful for body. [4] No direct relationship found between the use of benzalkonium chloride as preservative below 0.1% in nasal sprays and drug-induced rhinitis. [5] Now it is identified by some researchers that benzalkonium chloride should be avoided in nasal sprays. Benzalkonium chloride solutions act as biocidal agents with a prolong duration of action. They are strongly active against some viruses, bacteria, fungi, and protozoa. Bacterial spores are considered to be more resistant. Benzalkonium chloride solutions have bacteriostatic and bactericidal properties according to their concentration. Sensitivity of gram positive bacteria is more than other. This activity is remaining same by variation in pH, but increases significantly with gradually increase in temperatures and exposure time slots.

Azelastine hydrochloride ophthalmic solution is relatively selective H_1 receptor antagonist for topical drug administration to eyes. Azelastine hydrochloride has an antihistaminic therapeutic effects vulnerable providing immediate relief, mast cell stabilization providing early-phase intervention, and inhibition of expression and activation of anti-inflammatory mediators which characterize the late phase of the immune reaction. [6] Its chemical name is $(\pm)$ -1-(2H)-phthalazinone,4-[(4-chlorophenyl)methyl]-2-(hexahydro-1-methyl-1H-azepin-4-yl)-, mono hydrochloride. Its molecular formula is $C_{22}H_{24}ClN_3O \cdot HCl$ with the above chemical structure.

A number of analytical methods have been developed and reported for the estimation of benzalkonium chloride in various products including ophthalmic preparations. [7-22] But there was no method available for estimation of benzalkonium chloride in azelastine hydrochloride ophthalmic pharmaceutical formulations. Several HPLC and UV methods have been developed for the estimation of benzalkonium chloride in the past. The quantification of benzalkonium chloride by using these previously available methods in ophthalmic solutions was not suitable for our product of interest. As our ophthalmic formulation contained azelastine hydrochloride as an active ingredient and other excipients, injection at high

concentration was not suggested with the CN column. The suitable ideal method should have a high degree of sensitivity of detector, optimized sample concentration to maintain column robustness and low run-time analysis. The main objective of this work was analytical development and validation of method for estimation of total benzalkonium chloride content in azelastine hydrochloride pharmaceutical ophthalmic formulation by reverse phase high performance chromatography.

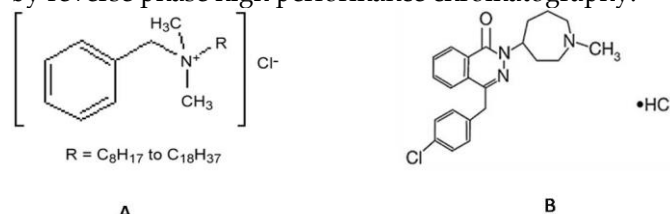


Fig. 1: Chemical structure of (A) benzalkonium chloride (B) azelastine hydrochloride

MATERIALS AND METHODS

Reagent and chemicals

Benzalkonium chloride 50% aqueous solution with 50% purity as a standard solution was obtained from fisher scientific, Mumbai. HPLC grade acetonitrile, Water (AR grade), hydrochloric acid were obtained from Merck, Mumbai. Hydrogen peroxide solution, Tri ethyl amine, sodium hydroxide pellets and ortho-phosphoric acid were obtained from fisher scientific, Mumbai. All reagents were used without any further purification. Sample received from Sun Pharma, Centaur pharmaceutical as a gift samples and from local pharmacy. All components which were obtained from commercial sources and used as received.

Equipment

Shimadzu LC-2010 HPLC system equipped with UV detector, quaternary solvent delivery pump, column thermostat, automatic sample injector using LC solution software as data acquisition tool was used as analytical technique for the estimation of total benzalkonium chloride content in azelastine pharmaceutical ophthalmic formulation.

Chromatographic condition

Analysis was performed at UV detection at 210 nm and the injection volume was 20-30 μ L. The HPLC equipped with maintaining column oven temperature with 25°C. The reverse phase column cosmosil C18, 250 mm length $\times$ 4.6 mm I.D. $\times$ 5 μ m particle size was used with a flow rate of 1.5 ml/minute for optimization of chromatographic conditions.

Preparation of buffer pH 5.0

A volume of 5.0 ml of ortho phosphoric acid was dissolved in 1000 ml of water and 1.0 ml of triethyl amine was added to this solution. The pH of buffer was adjusted to 5.0 with 5 N NaOH solution.

Preparation of mobile phase

Buffer and acetonitrile was mixed in the ratio of 55: 45% v/v, degassed and filtered by 0.45 μ membrane filter. The flow rate of pump for mobile phase was 1.5 ml/minute.

Preparation of standard solution

Standard solution having theoretical concentration of 50µg/ml of benzalkonium chloride was prepared using diluent.

Preparation of sample solution

Sample solution was prepared of pharmaceutical ophthalmic formulation containing final concentration of 50µg/ml benzalkonium chloride was prepared by using diluent.

Evaluation of System suitability

As per pharmacopoeia system suitability was the important part of method validation. This test was used to assure the repeatability and reproducibility of liquid chromatographic system at the time of real time analysis. To establish system suitability evaluation, standard solution of benzalkonium chloride injected in five replicates and the system suitability check were done as follows.

- 1) The %RSD of the total peak area of all homologs peaks should be not more than 2.0.
- 2) The theoretical plates of all peaks should be not less than 2000.
- 3) The tailing factor of all peaks should be not more than 2.5.

Method Validation

The method validation [23-24] for estimation of total benzalkonium chloride content was performed as per ICH Q2R1 guideline by determining the parameters: Specificity, linearity, precision and accuracy or recovery.

Specificity

Specificity is the ability to determine the analyte of interest in the presence of drug components which may be expected to be present. Each of the solution like diluent, standard solution and sample solution were injected on to HPLC equipped with detector and chromatograms were recorded. The specificity of method was determined by analyzing the various solutions like diluent, standard solution of benzalkonium chloride, placebo solution and ophthalmic sample solution of 100% level.

Instrument Precision

A standard solution of benzalkonium chloride in diluent having concentration 50µg/ml was prepared and injected in five replicates to establish system precision. Different parameters like retention time, tailing factor, theoretical plates and peak areas of all homolog's peaks were evaluated.

Method Precision

The precision of an analytical procedure describes the closeness of agreement between a series of measurements obtained from multiple sample preparations of the same homogeneous sample under the predefined certain conditions. Six sample solutions prepared for analysis of total benzalkonium chloride content for establishment of method precision and each sample was injected in duplicate. For each sample, chromatograms were recorded. The %RSD of six assay

determination of total benzalkonium content of sample preparation was calculated and found less than 2.0.

Accuracy/Recovery

The accuracy of an analytical quantitative method expresses the closeness of agreement between the accepted reference value and the value found. Accuracy of method is defined as the closeness of measured value to the true value for sample. Accuracy of a HPLC method was carried out at three different level i.e. $\pm 30\%$ of level claim so that 70%, 100% and 130% of benzalkonium chloride. The known quantity of benzalkonium chloride is analyzed at all three levels. Three samples at three concentrations (total nine determinations) were prepared at each concentration level and injected in duplicate.

Linearity/Range

The linearity of an analytical method is to establish direct correlation between concentration of analyte in the sample and detector response. [25] Linearity of detector with respect to different concentrations of benzalkonium chloride was studied by preparing the diluted standard solutions from stock standard solution. Linearity was performed to verify detector response in minimum of five concentration level between $\pm 20\%$ of 100% level concentration. Standard solutions of minimum five concentrations were injected in duplicate. A standard calibration curve was constructed between concentration and peak area of analyte. As an outcome of linearity slope, Y- intercept and correlation coefficient have been calculated.

RESULTS AND DISCUSSION

Development of method and Optimization of chromatographic conditions, sample Preparation

The primary and ultimate objective of this study was estimation of total benzalkonium chloride content in azelastine hydrochloride pharmaceutical ophthalmic solution. During the literature search, it has been found that various chromatographic methods for benzalkonium chloride determination have been reported. With support of these methods, various types of reverse phase columns have been used like C8, C12, C18 and CN columns. Different types of packing of columns like L1, L10 or CN packing were used for primary determination of benzalkonium chloride by HPLC. Aqueous buffer with arrange of pH 3.0 to 6.5 were used with acetonitrile or methanol in different ratio in mobile phase. [26] Significant effects of various factors were analyzed for separation of benzalkonium chloride homologs for quantification. [27] The effects of each approach like various column types, flow rate, mobile phase ratio and column oven temperature and different wavelength were summarized. After optimization of various column and buffer with different pH range with mobile phase consisting of acetonitrile and buffer, finally we have found suitable cosmosil column 250 mm $\times$ 4.6 mm $\times$ 5µm using mobile phase comprising of acetonitrile: buffer.

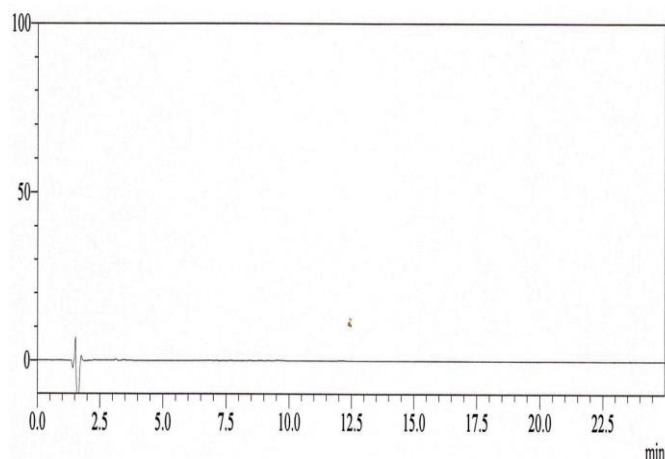


Fig. 2: Representative chromatogram for mobile phase

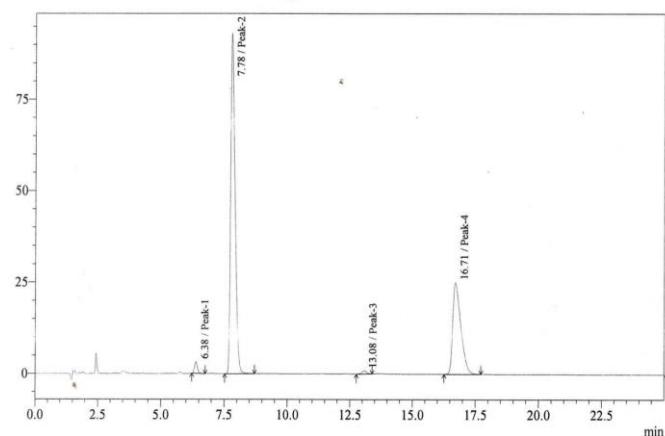


Fig. 3: chromatogram for benzalkonium chloride standard solution

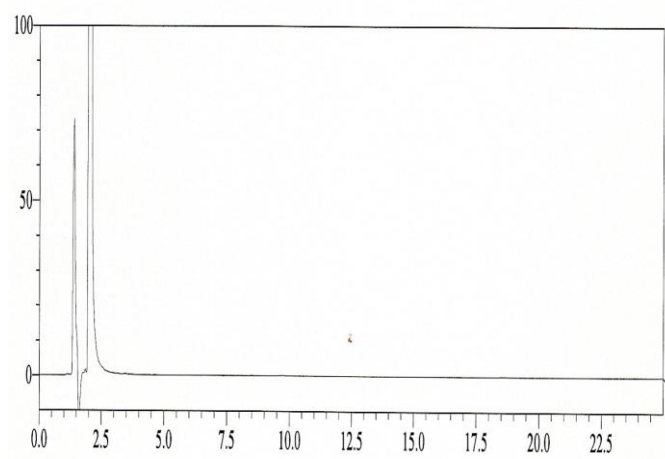


Fig. 4: Representative chromatogram of placebo solution

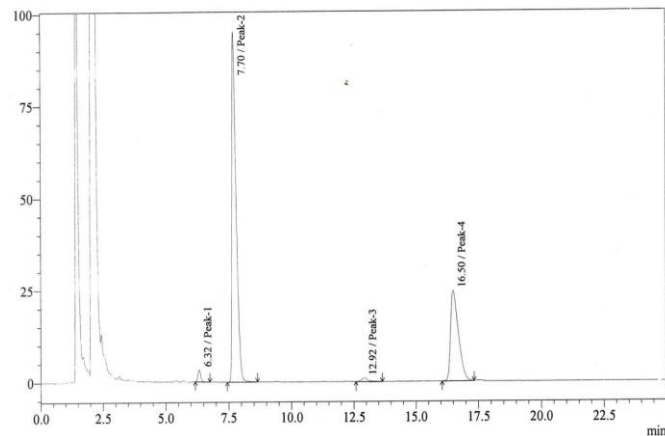


Fig. 5: Representative Chromatogram for Sample solution

Table 1: Evaluation of System suitability parameters for standard solution

Parameters	Acceptance Criteria	BKC Peak1	BKC Peak2	BKC Peak3	BKC Peak4
Retention Time	NA	6.15	7.49	12.54	16.02
Peak area Response	NA	26632	1176049	13277	585666.8
Theoretical plates	≥2000	13180	8595	16888	11996
Tailing factor	≤2.5	1.222	2.016	1.09	1.806
Relative Retention Time	NA	0.82	1.00	1.67	2.14
Resolution	NA	0.00	4.98	14.23	7.16

Table 2: Instrument precision

Injection Repeatability					
Injecti ons	Peak Area 1	Peak Area 2	Peak Area 3	Peak Area 4	Sum
1	25660	1123447	12444	557280	1718831
2	25536	1122972	12616	557055	1718179
3	25604	1121637	13400	556833	1717474
4	25606	1121712	17610	556977	1721905
5	25114	1120840	16444	556967	1719365
		Mean			1719151
		SD			1694.832
		%RSD			0.10

Table 3: Method precision

S. No.	Sample Name	% Assay Value
1	MP-1	100.3
2	MP-2	100.4
3	MP-3	100.6
4	MP-4	100.7
5	MP-5	100.0
6	MP-6	100.2
	Mean	100.4
	RSD	0.26

Table 4: Linearity parameter for total benzalkonium chloride

S. No.	Parameters	Result
1	Correlation Coefficient	0.999
2	Slope	36052.121
3	Intercept	4391.800

Table 5: Linearity parameters for individual benzalkonium chloride homologs

Parameters	BKC Peak1	BKC Peak2	BKC Peak3	BKC Peak4
Correlation Coefficient	0.999	0.999	0.995	0.999
Slope	534.505	23463.980	281.455	11772.202
Intercept	-7.400	7100.800	390.400	-2311.600

Optimization of sample preparation was also a difficult task due to complex composition of pharmaceutical ophthalmic formulation. Due to the property of sample, aqueous diluting solution of sample preparation created foaming and organic mixed diluents created the precipitation of some excipients. Different diluents were prepared and tested, the mobile phase was found as a suitable diluent. Also a mixture of water and acetonitrile in the ratio of 55:45 was found to be the best diluent for getting good recovery of total benzalkonium chloride as per ICH guideline criteria. By using this type of sample preparation, recovery results obtained between 98% to 102%. Typical chromatograms were

obtained for mobile phase, standard solution, placebo and a sample solution containing Azelastine active drug are presented as shown in figure 2, 3, 4 and 5. Four peaks of benzalkonium chloride were found in chromatograms obtained from both standard solution of benzalkonium chloride and sample solution.

Method Validation

System Suitability

The system suitability was performed to for verify the chromatograph at the time of operation by evaluation of chromatographic parameters from five replicate injection of standard solution of benzalkonium chloride (50µg/ml in diluent). The results is calculated for each homolog peak of benzalkonium chloride and found acceptable as shown in table 1.

Specificity

Specificity of analytical method is its capability to accurately measure and specifically the analyte in the presence of drug components, Specificity of method was determined by analysis of diluent, standard solution, placebo solution and sample solution. No significance interference was observed at the retention time of homolog peaks of benzalkonium chloride from other sample components so that it is demonstrated that method was specific for quantitative analysis of total benzalkonium chloride content in azelastine hydrochloride pharmaceutical ophthalmic formulation as shown in figure 2, 3, 4 and 5.

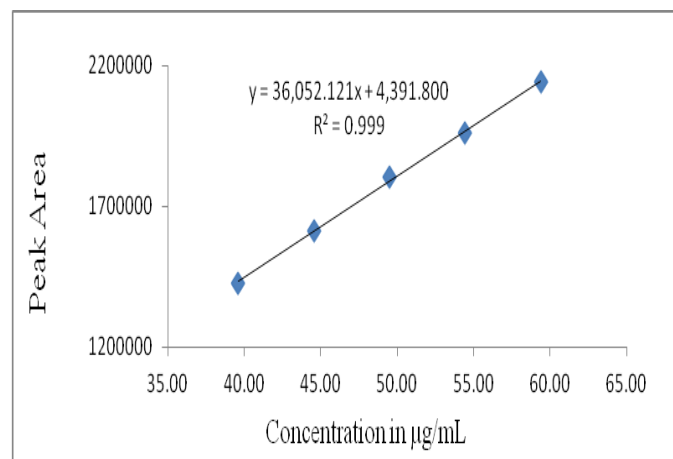


Fig. 6: Calibration curve of cumulative peak area of benzalkonium chloride

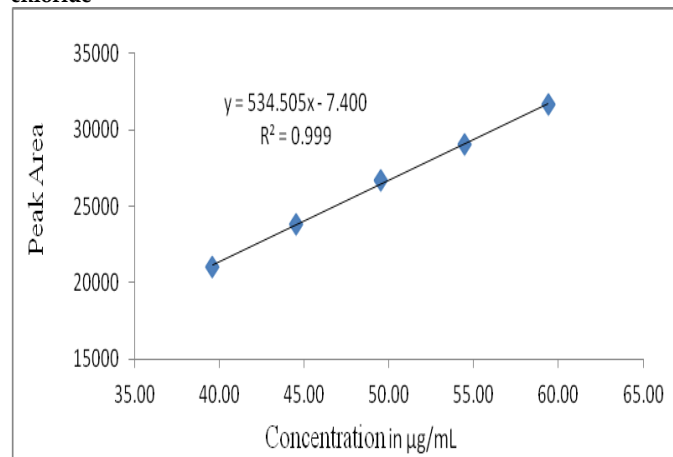


Fig. 7: Linearity curve for benzalkonium chloride peak 1

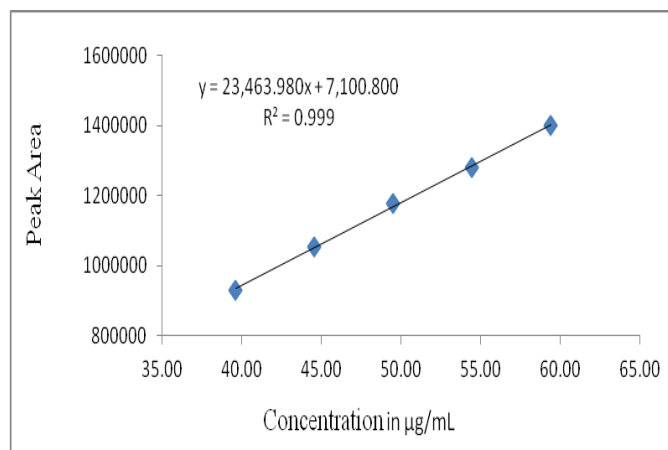


Fig. 8: Linearity curve for benzalkonium chloride peak 2

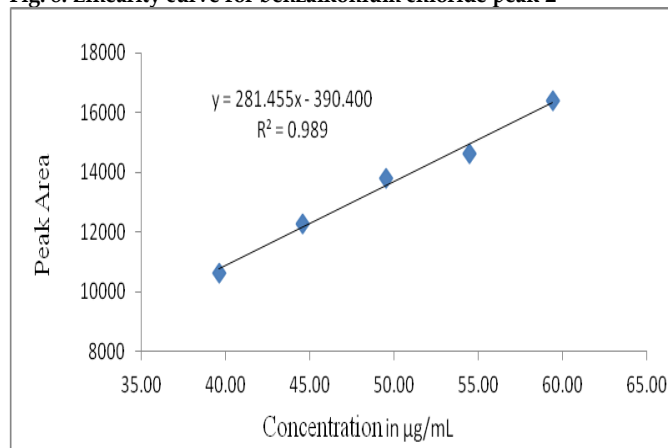


Fig. 9: Linearity curve for benzalkonium chloride peak 3

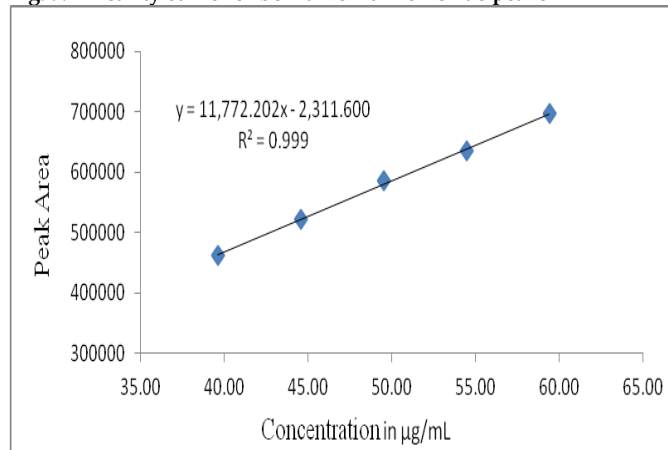


Fig. 10: Linearity curve for benzalkonium chloride peak 4

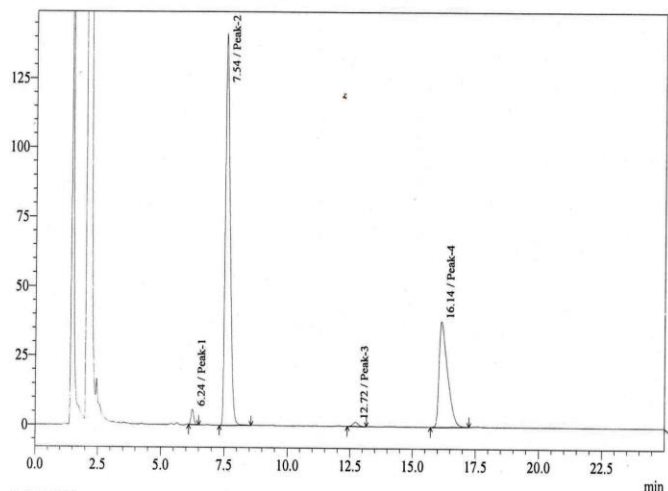


Fig. 11: Chromatogram of accuracy at 70% level

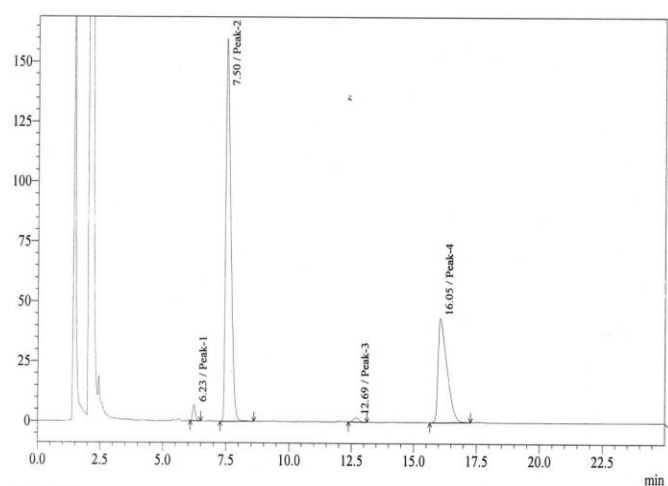


Fig. 12: Chromatogram of accuracy at 100% level

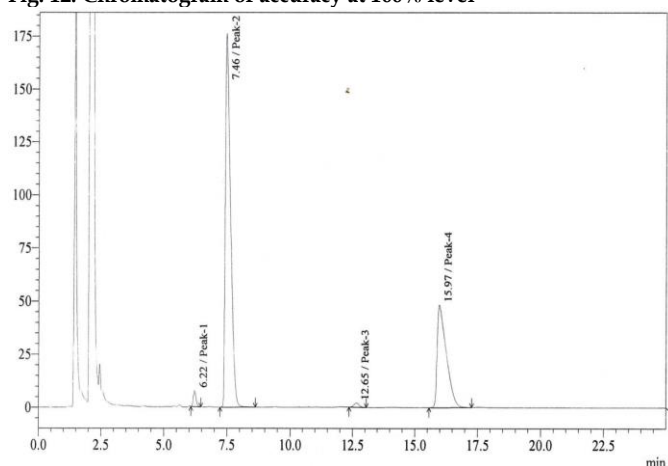


Fig. 13: Chromatogram of accuracy at 130% level

Table 6: Method accuracy for estimation of total benzalkonium chloride content

Theoretical		Experimental	Recovery (%)	Average (%)	RSD	Overall RSD
Accuracy level (%)	concentration (actual amount added in µg/ml)	Concentration Actual amount recovered in (µg/ml)				
70	32.69	32.17	98.4	99.0	0.74	0.80
		32.27	98.7			
		32.63	99.8			
		46.27	99.1			
100	46.70	47.35	101.4	100.4	1.19	
		47.10	100.8			
		61.53	101.3			
130	60.71	61.65	101.5	101.1	0.47	
		61.08	100.6			

Precision

Instrument Precision was established by injecting the standard solution in five replicates. The %RSD has been calculated for peak area response and retention time which were found under the acceptance limit as shown in table 2. Instrument precision demonstrates that instrument is capable of repeatability at time of analysis. The method precision was performed from six sample prepared and injected in duplicate and % RSD was obtained within acceptable limit ≤ 2 as shown table 3. Hence, the method is reproducible. The low %RSD

indicates that the method is reproducible and precise for quantification of benzalkonium chloride content as a preservative in this ophthalmic solution.

Linearity

The linearity of a method reveals the linear relationship of response against the selected concentration of analyte. Linearity of method was established as linear regression analysis with least square method for benzalkonium chloride. Five standard solutions containing benzalkonium chloride comprises of 80%, 90%, 100%, 110% and 120% of 100% target level corresponding to 40µg/ml to 60µg/ml. The linearity curve was obtained by plotting concentration of benzalkonium chloride standard solution versus the detector response. Regression line was established by statistical least squares method. A correlation coefficient of 0.999 was obtained as shown in figure 6 and results are discussed in table 4 for total benzalkonium chloride. The statistical results for all four individual homologs of benzalkonium chloride were shown in table 5 and figure 7, 8, 9, 10.

Accuracy/Recovery

Method accuracy was evaluated by preparing the sample solution at 70%, 100% and 130% level of total BKC labeled claim i.e. 35.69µg/ml, 46.74µg/ml and 60.71µg/ml. Recovery calculated for each level are 99.0% 100.4 and 101.1% and %RSD was calculated 0.74%, 1.19% and 0.47% respectively and overall RSD was calculated 0.80% as shown table 6. The results of recovery studies indicated that the analytical method is accurate for estimation of total benzalkonium chloride content in azelastine pharmaceutical ophthalmic formulations. Typical chromatogram is shown in figure 11, 12, 13.

The proposed chromatographic method was simple, specific, precise, linear, accurate and rapid for estimation of total benzalkonium chloride content as a preservative in azelastine pharmaceutical ophthalmic formulation. Newly developed and validated method differs from existing methods with respect to column, mobile phase composition, retention time and separation of analytes. The validation study of developed method described that this method was considerable estimation of total benzalkonium chloride content in azelastine pharmaceutical formulations.

REFERENCES

- Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. Progress in retinal and eye research. 2010 Jul 31;29(4):312-34.
- United States Pharmacopeia, USP 40-NF35. 2017; Benzalkonium chloride: 7531-7533.
- Liu J, Lu GW, Sandoval M, Ciringh Y, Xue G, Jaeger D, Kompanik K, Jiao J, Gelotte KM. Determination of benzalkonium chloride partition in micelle solutions using ultrafiltration method. AAPS PharmSciTech. 2009 Dec 1;10(4):1216-23.
- Nelson L, Goldfrank L. Goldfrank's Toxicologic Emergencies, 9 ed., McGraw-Hill Medical, 2011.
- Marple B, Roland P, Benninger M. Safety review of benzalkonium chloride used as a preservative in intranasal

- solutions: an overview of conflicting data and opinions. Otolaryngology-Head and Neck Surgery. 2004 Jan 31;130(1):131-41.
6. Azelastine Hydrochloride Ophthalmic Solution, 0.05%, OPTIVAR™, http://www.accessdata.fda.gov/drugsatfda_docs/label/2000/211271bl.pdf, 2000.
 7. Agarwal A, Tiwari S, Nagariya K. Method development and its validation for quantitative simultaneous determination of Latanoprost, Timolol and Benzalkonium chloride in ophthalmic solution by RP-HPLC. Journal of Drug Delivery and Therapeutics. 2013 Mar 14; 3(2):26-30.
 8. Al-Fakhory AA, Al-Kalak I, Al-Khatib MJ. Chromatographic Determination of Total Benzalkonium Chloride [BAC] in Some of Ophthalmic Preparations by HPLC. Damascus University Journal for Basic and Applied Sciences. 2014;30(2):155-71.
 9. Chiapetta SC, de Oliveira ÉC, Olivier BC, Mercante LA, Henriques DM, Pereira Netto AD. Intralaboratory validation, comparison and application of HPLC-UV-DAD methods for simultaneous determination of benzalkonium chloride, chlorexidine digluconate and triclosan. Journal of the Brazilian Chemical Society. 2011 Oct;22(10):1913-20.
 10. Dudkiewicz-Wilczyńska J, Tautt J, Roman I. Application of the HPLC method for benzalkonium chloride determination in aerosol preparations. Journal of pharmaceutical and biomedical analysis. 2004 Mar 10;34(5):909-20.
 11. Gaber M, Shawish HM, Khedr AM, Abed-Almonem KI. Determination of benzalkonium chloride preservative in pharmaceutical formulation of eye and ear drops using new potentiometric sensors. Materials Science and Engineering: C. 2012 Dec 1;32(8):2299-305.
 12. Jovović M, Kostić N, Jančić-Stojanović B, Malenović A. Investigation of tropicamide and benzalkonium chloride stability using liquid chromatography. Journal of Liquid Chromatography & Related Technologies. 2012 Jan 15;35(2):231-9.
 13. Kapakayala SV, Mubeen G, Kimbahune R. Reverse Phase-High Performance Liquid Chromatographic Method for Quantitation of Epinastine Hydrochloride in Eye Drops. Indo American Journal of Pharm Research. 2013;3(9):7541-8.
 14. Kostić DA, Mitić SS, Nasković DČ, Zarubica AR, Mitic MN. Determination of benzalkonium chloride in nasal drops by high-performance liquid chromatography. Journal of Chemistry. 2012 Apr 3;9(3):1599-604.
 15. Mehta J, Patidar K, Vyas N. Development and validation of a precise method for determination of benzalkonium chloride (BKC) preservative, in pharmaceutical formulation of latanoprost eye drops. Journal of Chemistry. 2010;7(1):11-20.
 16. Rao PV, Venkatesh P, Kumar MR. Validated stability indicating UPLC method for the estimation of benzalkonium chloride in ketorolac tromethamine ophthalmic solution. International Journal of Chromatographic Science. 2013;3(1):5-9.
 17. Santos M, Li M, Rustum AM. A single RP-LC method for the determination of benzalkonium chloride and its potential impurities in benzalkonium chloride raw material. Chromatographia. 2010 Mar 1;71(5-6):499-503.
 18. Shaikh KA, Patil AT. Stability-indicating HPLC method for the determination of mometasone furoate, oxymetazoline, phenyl ethanol and benzalkonium chloride in nasal spray solution. Journal of Trace Analysis in Food and Drugs. 2013;1:14-21.
 19. Shen Y, Xu SJ, Wang SC, Tu JS. Determination of benzalkonium chloride in viscous ophthalmic drops of azithromycin by high-performance liquid chromatography. Journal of Zhejiang University-Science B. 2009 Dec 1;10(12):877-82.
 20. Trivedi HK, Patel MC. Development and Validation of a Precise and Stability Indicating LC Method for the Determination of Benzalkonium Chloride in Pharmaceutical Formulation Using an Experimental Design. Journal of Chemistry. 2010;7(4):1514-22.
 21. Trivedi RK, Challa S, Patel MC, Trivedi DR, Chatrabhuji PM. A Rapid, Stability-Indicating RP-UPLC Method for the Simultaneous Determination of Fluticasone Furoate and Benzalkonium Chloride in a Pulmonary Drug Product. Chemical Science Transactions. 2013;2(4):1184-91.
 22. Labranche LP, Dumont SN, Levesque S, Carrier A. Rapid determination of total benzalkonium chloride content in ophthalmic formulation. Journal of pharmaceutical and biomedical analysis. 2007 Feb 19;43(3):989-93.
 23. International conference of Harmonization (2005) ICH harmonized tripartite guideline validation of analytical procedures: text and methodology Q2(R1) ICH, Geneva, Nov 2005.
 24. Daravath B, Reddy GS, Kamarapu SK. Development and validation of RP-HPLC for simultaneous estimation of Chlorpheniramine maleate and Diethylcarbamazine citrate in pharmaceutical dosage forms. Asian J Pharm Clin Res. 2014; 7(3):98-102.
 25. Katakam P, Sireesha KR. Stability indicating HPLC method for simultaneous determination of chloramphenicol and prednisolone acetate in bulk and formulations. Asian journal of pharmaceutical and clinical research. 2012;5(3):182-5.
 26. United States Pharmacopeia, USP 40-NF35, 2017. Benzalkonium chloride solution: 7333-7335.
 27. AlAani H, AlNukkary Y. Determination of Benzalkonium Chloride in Ophthalmic Solutions by Stability-Indicating HPLC Method: Application to a Stability Study. J Applied Pharm Sci. 2016; 6: 080-089.

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