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

Pratik Kumar Gupta*, Vibha Chaturvedi

Department of Chemistry, Mewar University, NH-79, Gangrar, Chhitorgarh-312901, Rajasthan, India

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 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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*Corresponding author: Mr. Pratik Kumar Gupta
Address: Department of Chemistry, Mewar University, NH-79, Gangrar, Chhitorgarh-312901, Rajasthan, India
Tel.: +91 9654002994
E-mail: pkpipraiya@gmail.com
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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 alkylbenzyldimethylammonium chlorides containing the chemical formula \([C_6H_{15}CH_2N(CH_3)R]Cl\), where R is an alkyl group changeable from \(C_6H_{17}\) 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 be 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 H1 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 (±)-1-(2H)-phthalazinone,4-[(4chlorophenyl)methyl]-2-(hexahydro-1-methyl-1H-azepin-4-yl)-,mono hydrochloride. Its molecular formula is \(C_{22}H_{35}ClN_2O\cdot HCl\) 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] 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.

![Chemical structure of (A) benzalkonium chloride (B) azelastine hydrochloride](image)

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 scientic, 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 scientic, Mumbai. All regents 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 20-30µL. The HPLC equipped with maintaining column oven temperature with 25°C. The reverse phase column cosmosil C18, 250 mm length x 4.6 mm I.D. x 5µm 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 0.45µ 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 a three different level i.e. ± 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 ± 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 × 4.6 mm × 5µm using mobile phase comprising of acetonitrile: buffer.
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

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  | 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                        | 253114      | 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        | 103.6         |
| 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            | 3665.2212      |
| 3      | Intercept        | 4391.8000      |

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 |

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
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 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.

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

\[
\begin{align*}
\text{Peak Area} & = 220000 - 200000 + 170000 - 150000 + 120000 - 100000 + 80000 - 60000 + 40000 - 20000 \\
& \quad \left(35.00, 40.00, 45.00, 50.00, 55.00, 60.00, 65.00\right) \\
\text{Concentration in µg/mL} & \quad y = 23,463.380x + 7,100.800 \\
R^2 & = 0.999
\end{align*}
\]

**Fig. 7: Linearity curve for benzalkonium chloride peak 1**

\[
\begin{align*}
\text{Peak Area} & = 35000 - 33000 + 31000 - 29000 + 27000 - 25000 + 23000 - 21000 + 19000 - 17000 \\
& \quad \left(35.00, 40.00, 45.00, 50.00, 55.00, 60.00, 65.00\right) \\
\text{Concentration in µg/mL} & \quad y = 534,505x - 7,400 \\
R^2 & = 0.999
\end{align*}
\]

**Fig. 8: Linearity curve for benzalkonium chloride peak 2**

\[
\begin{align*}
\text{Peak Area} & = 160000 - 140000 + 120000 - 100000 + 80000 - 60000 + 40000 - 20000 \\
& \quad \left(35.00, 40.00, 45.00, 50.00, 55.00, 60.00, 65.00\right) \\
\text{Concentration in µg/mL} & \quad y = 281,455x - 390,400 \\
R^2 & = 0.989
\end{align*}
\]

**Fig. 9: Linearity curve for benzalkonium chloride peak 3**

\[
\begin{align*}
\text{Peak Area} & = 800000 - 700000 + 600000 - 500000 + 400000 - 300000 + 200000 - 100000 \\
& \quad \left(35.00, 40.00, 45.00, 50.00, 55.00, 60.00, 65.00\right) \\
\text{Concentration in µg/mL} & \quad y = 11,772,202x - 3,311,600 \\
R^2 & = 0.999
\end{align*}
\]

**Fig. 10: Linearity curve for benzalkonium chloride peak 4**

**Fig. 11: Chromatogram of accuracy at 70% level**
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 formulations. 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.

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