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Analytical Method Development and Validation of Preservative Benzalkonium Chloride in Ciprofloxacin Eye Drops by HPLC

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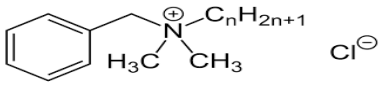
Abstract

An accurate, precise, linear, specific and cost effective simple HPLC method has been developed and validated for estimation of Benzalkonium Chloride. Separation of the Preservative was achieved on a L10 column (Dimension: 15 cm × 4.6 mm, 5 µm particle size) using a mobile phase consisting of a mixture of Phosphate buffer (PH 5.5) and acetonitrile (40:60, v/v). The flow rate and detection wavelength were 1 mL/min and 210 nm respectively. The linearity was found in the concentration of 0.05, 0.08, 0.10, 0.12, 0.15, mg/mL as 50% solution of Benzalkonium Chloride with a correlation coefficient (R2) of 0.999. The retention time of Benzalkonium Chloride-1 and Benzalkonium Chloride-2 were 5.965 and 6.993 minutes respectively. The predicted method was validated as per the International Council for Harmonization Guidelines (ICH) for the parameters: Linearity, Accuracy, Precision, Robustness and Specificity. This method can be used for routine analysis of quality control of Benzalkonium Chloride in Ophthalmic dosage form.

Keywords: Benzalkonium chloride; Ciprofloxacin eye drops; Potency; Method validation; Preservative; High Performance Liquid Chromatography (HPLC)

Introduction

Ophthalmic products are sterile aqueous or oily solutions or suspensions of one or more active materials and normally packed in suitable multi-dose containers which allow the instillation of consecutive drops of the preparation. During use and storage of ophthalmic preparations may lead to product spoilage or may induce serious ocular infections due to microbial contamination or proliferation [1]. To protect of these multi-dose preparations are commonly used preservatives [1]. Benzalkonium Chloride (BKC), a mixture of N-Alkyl-N-benzyl-N, N-dimethylammonium chloride (Figure 1) which is commonly used as preservative and various dosage forms including ophthalmic preparations [2]. BKC is a mixture of alkyls, including all or some of the group beginning with n-C_8H_{17} and extending through higher homologs, with n-$C_{12}H_{25}$, n$C_{14}H_{29}$, and n-$C_{16}H_{33}$ composing the major portion. It is presented as yellowish-white powder or gelatinous, a white or yellowish-white fragments. Benzalkonium chloride is hygroscopic. On heating it forms a clear molten mass (EP 0372). Benzalkonium Chloride is an effective fungicidal and bactericidal agent that helps to reduce the growth of organisms in multi-dose containers [3]. BKC was first introduced in the 1910s as a germicide and became vastly used in the 1940s. BKC was first used in the 1940s for preserve hard contact lens solution in the ophthalmic industry. Since then, it has been used as antiglaucoma drugs to over the counter synthetic tear solutions [4]. BKC seems to be the key preservative in optic preparations on the EU market. Nearly 74% of optic preparations have BKC as a preservative [5]. BKC also used as an antimicrobial preservative in many drug products for nasal route of administration and in many presentations for respiration use authorized on EU markets. The concentration of BKC used in pharmaceutical preparations is 0.002% – 0.02%, but sometimes it could be up to 0.2% depends on various factors in ophthalmic formulations [2]. In some cases, the drug products that contain BKC are intended for oral, or mucosal, rectal, cutaneous, intramuscular, intra-articular, intravenous, subcutaneous, vaginal and auricular use. BKC has three prime types of use: as a cationic surfactant, phase transfer agent and a biocide in the chemical industry. BKC was form to be an efficient method of contraception [6]. Benzalkonium chloride is used for the treatment of superficial infections of the mouth and throat that containing by Lozenges [7]. BKC works by killing microorganisms and inhibiting their future growth, and for this reason frequently appears as an ingredient in antibacterial hand wipes, antiseptic creams, anti-itch ointments and ophthalmic preparation. The US-FDA specifies that the safe and efficient concentrations for BKC are 0.1 to 0.2% in first aid products. Various analytical methods have been developed for the estimation of BKC in ophthalmic preparations [8-19]. However, the described HPLC methods were limited to a number of ophthalmic preparations. Therefore, the aim of this study was to develop and validate a cost effective simple HPLC method for estimation of Preservative Benzalkonium Chloride in Ciprofloxacin Eye Drops.



n = 8, 10, 12, 14, 16, 18

Figure 1: Chemical structure of Benzalkonium chloride.

Materials and Methods

Chemicals and solvents

Ciprofloxacin eye drop was obtained from Lazz Pharma, Benzalkonium Chloride was obtained as gift sample from Sonali Scientific Store; Potassium Dihydrogen phosphate (AR grade) was

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Sigma Aldrich Germany; Sodium Hydroxide was Daejung Korea; Ortho-phosphoric acid (AR grade) was procured from Merck Germany; HPLC grade water was taken from Incepta Pharmaceuticals Ltd, Bangladesh.

Instrumentation

HPLC (Waters- Corporation, USA), Analytical balance (Mettler Toledo, Switzerland), Ultrasonic bath (Sonoswiss, Switzerland) and pH meter (Mettler Toledo, Switzerland).

Preparation of buffer solution

Dissolve 0.7 g of Sodium Hydroxide and 5.5 g potassium di-hydrogen phosphate in 1000 mL water. Adjust the pH to 5.5 with dilute phosphoric acid.

Preparation of mobile phase and diluent

Mixed 40 volumes of buffer with 60 volumes of acetonitrile. The mixture was degassed in a sonicator for about 10 minutes and it was then filtered through 0.45 µm membrane filter under vacuum. The filtrate was stored at room temperature to use as mobile.

Preparation of standard solution

Weigh accurately about 100 mg of Benzalkonium Chloride Solution (50%) working standard and transfer into 50 mL volumetric flask with diluent and sonicate for 10 minutes. Transfer 10 ml into 100 mL volumetric flask volume with diluent upto the mark and mix well. Filter the solution through 0.2 µm disc filter and collect the solution into a clean and dry vial.

Preparation of sample solution

Direct Filter the solution through 0.2 µm disc filter and collect the solution into a clean and dry vial.

Chromatographic analysis

The analysis of the Preservative was carried out by Waters HPLC which contained a quaternary low-pressure gradient pump, PDA Detector equipped with temperature-controlled auto sampler and column oven. Chromatographic analysis was performed using L10 column with 150 × 4.6 mm internal diameter and 5 µm particle size. Isocratic elution with flow rate 1 mL per minute was selected. The detector was PDA and wavelength was set at 210 nm, column oven temperature 30 degree celcious and the injection volume was 50 µL with a run time of 10 minute. The mobile phase was prepared and degassed then sonicated for 10 minute before use. The column was stabled for 50 minute with the mobile phase flowing through the system. The column and HPLC system was kept at 30 degree Celsius temperature.

Chromatogram with working standard

Benzalkonium Chloride 50 % solution (100 mg) was weighed and transferred to a 50 mL volumetric flask, sonicated to dissolve with diluent and volume with diluent. It contained 0.1 mg of BKC in each mL of solution for 100% concentration. By this same way accurately weighed 200 mg and transferred into 100 mL volumetric flask for linearity stock solution and from this stock solution transferred 5 mL, 8 mL, 10 mL, 12 mL, 15 mL into 100 mL volumetric flask and volume up to the mark with diluent for the concentration of 50%, 80%, 100%, 120%, and 150% respectively. Each of the solution (50 µL) was injected by auto injector into the column at a flow rate 1 mL per minute of mobile phase and the corresponding chromatogram was recorded (Figure 2). It is explicit from the Figure 2 that the chromatogram was quite good and it could be used for qualitative and quantitative analysis of Benzalkonium Chloride-1 and Benzalkonium Chloride-2. Retention time of the chromatogram was ascertained from the replicates and it was found as 5.965 and 6.993 minutes.

Calibration plot

The calibration graph was constructed by plotting concentrations of the drug against area (µV) of the chromatogram at RT=5.965 and 6.993 min for Benzalkonium Chloride-1 and Benzalkonium Chloride-2 and it was found linear (Figure 3). The regression equation for the curve was found as y=32468902.34093X-8066.55862 with correlation coefficient (R^2) 0.99996. It was used to estimate the amount of Benzalkonium Chloride.

Validation of the proposed method

The system suitability, specificity, linearity, accuracy, precision, Range and Robustness parameters of method validation were cultivated systematically to validate the raised HPLC method as per ICH guidelines.

System suitability: In validating the method, it was important to check system suitability which was done by the relative standard deviation calculation of the peak area of 6 replicates of standard [20]. Results and relevant discussion are presented in the results and discussion section.

Linearity: Linearity of the analytical method was judged by three studies regression analysis of Benzalkonium chloride without excipients, regression analysis of Benzalkonium chloride with fixed concentration of excipients and regression analysis of Benzalkonium chloride with different concentration of excipients.

Limit of Quantification (LOQ): LOQ was destined based on STDEV of response and slope method. Linearity was performed in the specified range of the reference sample solution concentration.

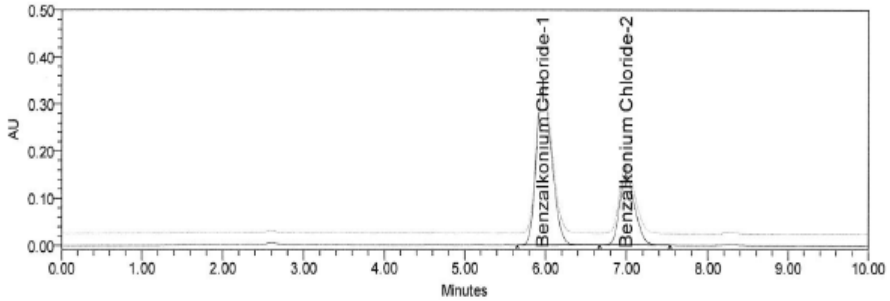

Figure 2: A Representative chromatogram of Benzalkonium Chloride-1 and Benzalkonium Chloride-2 (RT=5.965 and 6.993 min) under optimized conditions.

Linearity graph of concentration in mg/mL (X-axis) versus peak response (Y-axis) was plotted. LOQ was calculated using correlation coefficient, slope of regression line and standard deviation of regression line.

Limit of detection: LOD was destined based on STDEV of response and slope method. Linearity was performed in a specified range of reference sample solution concentration. Linearity graph of concentration in mg/mL (X-axis) *versus* peak response (Y-axis) was plotted. LOD was calculated by correlation coefficient, slope of regression line and standard deviation of regression line [21].

Range: The specified range is normally derived from linearity studies. Range has been calculated from the lower and upper concentration of analyte in the sample for which it has been demonstrated that the analytical procedure provides an acceptable degree of precision, linearity and accuracy.

Specificity: Specificity of the procedure was judged from assessing unequivocally the analyte in the presence of component i.e. excipients that are expected to be present in a dosage form. Regression equation was used to assess the content of analyte in the test sample (Figure 3).

Placebo effect: Placebo effect was studied by running the blank, placebo and active solution in HPLC.

Accuracy: In case of assay of the drug in the formulated product, accuracy of the method was determined first. To do so a blank matrix (Placebo); the excipients (all ingredients except Preservative as per formulation of Ciprofloxacin eye drop) simulated Benzalkonium Chloride Sample (excipients + preservative) (50%, 100% and 150%) were run separately in three replicates in the HPLC.

Precision: Precision of a method for validation purpose was judged from repeatability, intermediate precision and reproducibility. Repeatability precision was carried out by six determinations at the fixed level of test sample concentration in homogeneous solution. Intermediate precision was determined from the HPLC measurements in different days by different analysts using different equipment within the same laboratory. Reproducibility of the stated HPLC method was verified involving analysts, other than those involved in repeatability and intermediate precision experiments, where six determinations were executed immediately one after the other in a different laboratory [6].

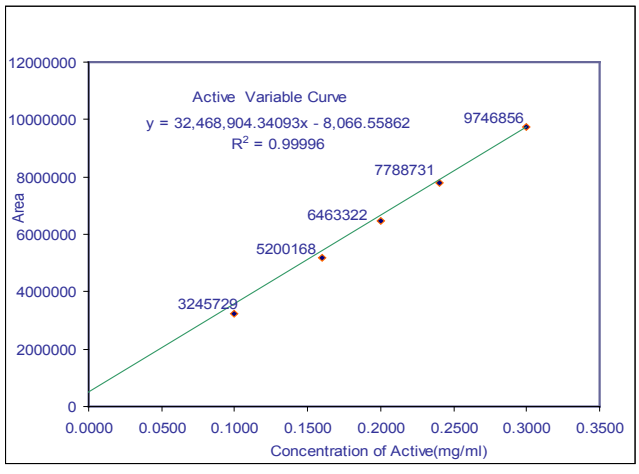

Figure 3: Calibration curve for Benzalkonium Chloride (BKC-1+BKC-2) only (Working standard).

Robustness: Robustness of the method was judged from the stability study of Benzalkonium Chloride sample solution at 30°C temperature (25°C – 35°C) at different time (0 hr, 24 hr) with time interval.

Results and Discussion

Benzalkonium Chloride is primarily used as a preservative and antimicrobial agent, and secondarily used as a surfactant. It works by killing microorganisms and inhibiting their future growth. People are working to find a suitable method for Benzalkonium Chloride quantification. In this paper a simple, cost effective and new method has been presented. This HPLC method was validated subsequently to assay the ophthalmic dosage form of Benzalkonium Chloride preservative.

System suitability

Standard solution was injected onto the HPLC system and chromatograms were recorded. The results are summarized in Tables 1-6. Linearity of analytical method was determined by performing studies regression analysis of Benzalkonium Chloride with different concentration. From the plot of the results the linear regression equation was obtained as: y=32468904.34093X-8066.55862 with different concentration of BKC and the response was linearly dependant on the concentration of BKC (Figure 3). The linearity of the regression line is also evident from correlation coefficient R^2 = 0.99998 (Tables 2 and 7). It is important to mention here that the proposed HPLC method for BKC estimation was found linear in the range of 0.05 to 0.15 mg/mL (Figure 3). LOD and LOQ were determined to be 0.0017 and 0.0052 respectively. The specificity of the method was reviewed by checking a standard (Preservative) solution of BKC, its eye drop, blank sample and placebo (excipients) materials. Sample of standard and eye drop showed peak BKC-1 and BKC-2 at retention time 5.965 and 6.99 minutes when run separately in HPLC, while blank and placebo did not show any peak at that RT value. These results indicated that BKC could be detected by the present method and it was also able to separate BKC from its excipients quantitatively (Table 2). Percent recovery of BKC in the presence and in the absence of excipients was found within the limit of ICH guideline and thus it means that the developed method is selective for quantification of BKC (Table 2) [22]. Accuracy was assessed using nine determinations over three different concentration levels covering the predetermined range (0.05-0.15 mg/mL) of analysis. And there were three replicates of each concentration (Tables 3 and

Table 1: Optimized chromatographic conditions Benzalkonium chloride.

Test	Condition
Mobile Phase	Acetonitrile : Buffer (60:40V/V), Isocratic
Diluent	Mobile phase
Column	L10 (Dimension: 15 cm × 4.6 mm), 5 µm
Column oven	30°C
Flow rate	1.0 mL/min
Detector	PDA
Injection volume	50 µL
Run time	10

Table 2: Results of specificity.

Sample Name	Purity Angle		Purity Threshold		Remarks
	BKC-1	BKC- 2	BKC-1	BKC-2	
Sample	0.183	0.165	0.246	0.263	Peak pure
Spiked Sample	0.158	0.161	0.260	0.294	Peak pure
Blank solution	-	-	-	-	No interfering peak

Table 3: Percent recovery of Benzalkonium Chloride from simulated tablet contents.

Sample No	Spiked level (%)	Amount added (mg)	Amount found (mg)	ICH Limit for % Recovery	% of Recovery	Mean (%)	Remarks
1	50	10.02	10.0	98% to 102%	99.9	99.8	The Method is accurate
2	50	10.03	10.0		99.8		
3	50	10.05	10.0		99.7		
4	100	20.23	20.3		100.3	100.4	
5	100	20.22	20.3		100.4		
6	100	20.21	20.3		100.4		
7	150	30.23	30.7		101.5	101.2	
8	150	30.25	30.6		101.1		
9	150	30.23	30.6		101.0		
Grand average (%)			100.4				
RSD(%) of 09 determinations			0.6				

Table 4: Relative standard deviation of six determinations of Benzalkonium Chloride contents (method Precision) in simulated tablet amount.

Sample	Concentration (mg/mL)	Peak area (µV)	% of Benzalkonium Chloride	RSD (%)	ICH limit of RSD (%)	Remarks
01	0.1	6479715	101.8	0.1	NMT 2.0	Method Precision of Benzalkonium Chloride measurements is complied
02	0.1	6483742	101.9			
03	0.1	6480282	101.8			
04	0.1	6489845	102.0			
05	0.1	6479783	101.8			
06	0.1	6482956	101.9			

Table 5: Relative standard deviation of six determinations of Benzalkonium chloride contents (Intermediate Precision) in simulated tablet amount.

Sample	Concentration (mg/mL)	Peak area (µV)	% of Benzalkonium Chloride	RSD (%)	ICH limit of RSD (%)	Remarks
01	0.1	6632161	98.8	1.1	NMT 2.0	Intermediate Precision of Benzalkonium Chloride measurements is complied
02	0.1	6592133	98.2			
03	0.1	6575686	98.0			
04	0.1	6595780	98.3			
05	0.1	6756952	100.7			
06	0.1	6575575	98.0			

Table 6a: Data for System Suitability of Benzalkonium chloride-1.

Injection Number	Retention Time (In minutes)	Peak Area	USP Plate Count	Tailing Factor
01	5.916	4448595	4364	1.24
02	5.919	4451563	4390	1.24
03	5.920	4453391	4393	1.25
04	5.922	4457853	4396	1.25
05	5.918	4457605	4373	1.24
06	5.920	4456271	4384	1.25
Average	5.919	4454213	4383	1.25
RSD (%)	0.04	0.08	NA	NA

Table 6b: Data for System Suitability of Benzalkonium chloride-2.

Injection Number	Retention Time (In minutes)	Peak Area	USP Plate Count	Tailing Factor
01	6.911	1979062	6518	1.24
02	6.915	1980283	6566	1.24
03	6.916	1980240	6583	1.24
04	6.919	1984273	6551	1.25
05	6.915	1984468	6559	1.25
06	6.918	1981168	6567	1.24
Average	6.916	1981582	6557	1.24
RSD (%)	0.04	0.11	NA	NA

4) from guideline. Thus, it was indicated that the proposed method was accurate for the analysis of the preservative BKC. Repeatability precision was carried out by six independent determinations of a fixed test concentration (0.1 mg/mL) of a homogeneous solution of BKC (Table 4). Values of RSD were calculated from these determinations and the obtained RSD value was reviewed to see whether it was within the limit (NMT 2%) of ICH guideline [ICH Harmonized, 2008]. In the present case, RSD was found as 0.1% which was within the limit (NMT 2%) of ICH guideline and hence the repeatability was compiled for the present method of analysis of Benzalkonium Chloride (Table 4) [8]. Similarly, it was found that the intermediate precision and system suitability criteria were as per ICH guideline (Tables 5 and 6) [22]. These determinations, it was found that the values of recovery for each estimation were within the range (98%-102%) of ICH percentage recovery [22]. The sample solution was allowed to stand at room temperature (20-25°C) for different time intervals (0, 24 hrs) to see the stability of BKC. The % assay result difference from initial value obtained 0.1 against the ICH limit (NMT 2%) which indicated that the working sample solution was stable for at least 24 hours. In the light of validation parameters results, it can be told that the developed method is valid for the estimation of BKC from the eye drop formulation.

Conclusion

This isocratic HPLC method was developed and validated for the analysis of Benzalkonium Chloride in ophthalmic dosage form. The developed method is less costly than the methods reported so far.

Table 7: Data for linearity.

Concentration in Percent	Name Of Injection	Concentration in mg/mL as 50%	Peak area		Mean		Statistical data
			Benzalkonium Chloride-1	Benzalkonium Chloride-2	Benzalkonium Chloride-1	Benzalkonium Chloride-2	
50	Injection 1	0.05	2248336	998626	2247981	997748	Corr. Coefficient :0.99998 y-intercept :-8066.55862 Slope : 32468904.3409X
	Injection 2		2248414	996170			
	Injection 3		2247193	998450			
80	Injection 1	0.08	3597100	1600979	3598827	1601341	
	Injection 2		3604626	1603286			
	Injection 3		3594756	1599757			
100	Injection 1	0.10	4468348	1989023	4471745	1991577	
	Injection 2		4472775	1992986			
	Injection 3		4474112	1992722			
120	Injection 1	0.12	5386702	2398412	5388916	2399812	
	Injection 2		5388932	2400627			
	Injection 3		5391115	2400395			
150	Injection 1	0.15	6740299	3005260	6742100	3004756	
	Injection 2		6737472	3001514			
	Injection 3		6748530	3007494			

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Competing Interests

Authors have declared that no competing interests exist.

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