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Determination of benzalkonium chloride in viscous ophthalmic drops of azithromycin by high-performance liquid chromatography*

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Abstract: A high-performance liquid chromatography (HPLC) system was used in the reversed phase mode for the determination of benzalkonium chloride (BKC) in azithromycin viscous ophthalmic drops. A Venusil-XBP(L)-C_{18} (150 mm×4.6 mm, 5 μm) column was used at 50 °C. The mobile phase consisted of a mixture of methanol-potassium phosphate (16:5, v/v). Two sample preparation methods were compared. The results suggested that, compared with an extraction procedure, a deproteinization procedure was much quicker and more convenient. Using the deproteinization procedure for sample preparation, calibration curves were linear in the range 5.0~50 μg/ml. The within-day and inter-day coefficients of variation were less than 10%. The average recoveries were determined as 96.70%, 98.52%, and 97.96% at concentrations of 10.0, 30.0, and 50.0 μg/ml, respectively. Variability in precision did not exceed 5%. In conclusion, this HPLC method using a simple sample treatment procedure appears suitable for monitoring BKC content in azithromycin viscous ophthalmic drops.

Key words: Benzalkonium chloride, High-performance liquid chromatography, Azithromycin, Viscous ophthalmic drops
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INTRODUCTION

Ophthalmic dosage formulations are the preferred way to treat ophthalmic diseases (Ali and Lehmussaari, 2006). There are two disadvantages associated with ophthalmic dosage formulations: low ocular bioavailability (Choy *et al*., 2008) and possible microbiological contamination arising from multiple dosing. To increase the precorneal retention of drugs, opthalmic drops are sometimes thickened with a gelling agent. Polymers, such as sodium hyaluronate, guar gum, gellan gum, hydroxyl methylcellulose, polyvinyl alcohol, hydroxypropyl methylcellulose, carboxyl methylcellulose, and polyacrylic acid derivatives (e.g., carbomers), have been used for improving ocular drug bioavailability (Aragona *et al*., 2002; Jiménez *et al*., 2007; Rozier *et al*., 1997; Tatavarti and Hoag, 2006; Winterton *et al*., 2007). To prevent the patient from administering microbiologically contaminated product to the eye, antimicrobial preservatives are added to the formulation. There is significant laboratory evidence that some preservatives can add to the potency of an antibiotic by increasing the speed of killing of potential pathogens and by lowering the minimal inhibitory concentration (MIC) of the commercial formulation for known pathogens (Samalonis, 2006).

Although several compounds can be used as ophthalmic preservatives, benzalkonium chloride (BKC), a quaternary ammonium compound, is commonly used (Labranche *et al*., 2007) because of its high potency. Azithromycin ophthalmic drops were developed to treat ocular bacterial infections. As a broad-spectrum antibacterial agent, azithromycin has ocular pharmacokinetic properties superior to those of other macrolides. Ocular pharmacokinetic

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results indicated that only a viscous solution of azithromycin can maintain a precorneal concentration above the MIC_{90} (MIC required to inhibit the growth of 90% of organisms) over 24 h (Rojsitthisak *et al*., 2005; Deutschle *et al*., 2006; Merianos, 1991; Prince *et al*., 1999). Our developed product contained sodium hyaluronate as the viscous agent, and BKC was chosen as the preservative.

BKC toxicity involving damage to the human nose epithelia and exacerbation of rhinitis has been reported previously (Merianos, 1991). However, the impact of BKC on the eye is not clear. BKC has been included in the US Food and Drug Administration (FDA) Inactive Ingredient Database (IID). Because of the long precorneal retention of BKC, the BKC content in viscous drops or product is usually no more than 0.008% (w/v) (Prince *et al*., 1999). It is important to monitor the content of BKC in opthalmic preparations to avoid toxicity concerns.

Many challenges are associated with the determination of BKC content in ophthalmic products or viscous solutions. Three key issues need to be addressed. Firstly, BKC is a mixture of alkylbenzyldimethylammonium chloride with the formula $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R is an alkyl group varying from C_8H_{17} to $C_{18}H_{37}$ (Rojsitthisak *et al*., 2005) (Fig.1). It was reported that homologs of C_{12} and C_{14} were the most common components suitable for ophthalmic formulations (Deutschle *et al*., 2006). A complete method should allow for the homologs of BKC to be quantified. Secondly, a suitable pretreatment is required to filter the high-performance liquid chromatography (HPLC) analyte solution of all polymeric agents. Ophthalmic viscous drops containing viscosity increasing agents injected directly into the HPLC without suitable pretreatment will foul the chromatographic column. Finally, the method must allow for BKC sensitivities well below the 0.008% level used in these formulations.

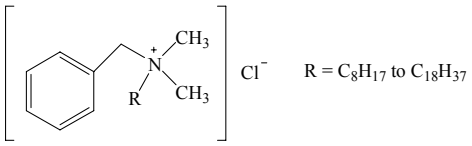

Fig.1 Representation of benzalkonium chloride

Several HPLC (Gomez-Gomar *et al*., 1990) and electrophoresis (Hou *et al*., 2002; Bernal *et al*., 1998) methods have been used for determining the qualitative and quantitative presence of BKC homologs in ophthalmic formulations. However, these methods are not suitable for ophthalmic gel or viscous solutions because they have low detection sensitivity and need long run-time sequences (in the range of 20~30 min) especially when using reversed-phase cyano (CN) columns. This type of column packing can be problematic with certain types of ophthalmic formulations. For example, formulations with sulfamide and sulfamide/morpholine as active ingredient and multiple excipients, when injected at high concentration, were not suitable for use with the CN stationary phase (Labranche *et al*., 2007).

The objective of the present study was to develop a method for routine determination of BKC in azithromycin viscous ophthalmic drops with convenient sample preparation and rapid analysis time using reversed-phase HPLC.

MATERIALS AND METHODS

Chemicals and reagents

BKC was obtained from Xuanguang Technology, Inc. (purity=99.5%; Nanjing, China). Sodium hyaluronate was purchased from Freda Biochem Co. Ltd. (Shandong, China). Azithromycin was a gift from Dongfeng Pharm. Co. Ltd. (Hebei, China). Methanol of HPLC grade was obtained from Shandong Yuwang Sci-Tech Co. Ltd., China. All other reagents were of analytical grade.

Equipment

The HPLC system consisted of a Waters 515 HPLC Pump, a Waters 2487 HPLC detector (Waters, USA) set at a wavelength of 208 nm (λ_{max}) and equipped with a Sanrui Chromatography Workstation (Sanrui Sci-Tech Co., Shanghai, China).

Chromatographic conditions

The samples were analyzed on a reversed phase Venusil-XBP(L)-C_{18} (150 mm×4.6 mm, 5 μm) column provided by Agela Technologies Inc. A precolumn (4 mm×100 mm) of the same material was also used. The mobile phase consisted of a mixture of methanol-4.0 mmol/L potassium phosphate (pH 3.0; 16:5, v/v), filtered and degassed before use and

pumped at a flow rate of 1.5 ml/min. The column thermostat was set at 50 °C on the column heater (Hanbang-Tech Co., Huaiyin, China). Under these experimental conditions the run time was 12 min. The United States Pharmacopeia (USP) method was used as a comparison to analyze whether the experimental method was fit for BKC determination.

Sample preparation

A primary aqueous stock solution of BKC (1.0 mg/ml) was prepared and diluted further with methanol-water (16:5, v/v) mixture to prepare secondary stock solutions (200.0 μg/ml). These secondary stock solutions were diluted further with mobile phase to produce spiking solutions with BKC concentrations of 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 μg/ml and sodium hyaluronate was added to each solution (equivalent to 60 times the amount of BKC).

Deproteinization procedure: To eliminate the influence of the polymer, the calibration standards were treated as follows: 1.0 ml aliquots of the BKC solutions were added to polypropylene tubes and 4.0 ml aliquots of methanol were added to precipitate the polymers in the eye drops. These tubes were vortexed for 2.0 min and centrifuged at 10000 r/min at 4 °C. The supernatant liquid was used to prepare the calibration standards at the time of the assay.

Extraction procedure: to extract BKC, 500 μl aliquots of spiking solutions were extracted with 5 ml of methanol-ethyl acetate (1:1, v/v) and the mixture was vortexed for 1 min and then centrifuged at 4000 r/min for 20 min at 4 °C. The upper layer (4 ml) was then removed and evaporated to dryness under reduced pressure (10 mmHg) at 37 °C. After drying the organic solvent, the residue was dissolved in the mixture of the mobile phase, and 20 μl was injected into the HPLC.

The two procedures generated a set of calibration standards with different concentrations of BKC. The tubes were stored frozen at −20 °C before use. These calibration standards were used to generate standard curves and were analyzed in validation runs to collect precision and accuracy data.

Limit of detection of BKC

A set of calibration standards with concentrations equivalent to 0.01, 0.10, and 1.0 μg/ml BKC was prepared as described above. The sample concentration with a signal-noise ratio (S/N) above 3 (0.01 μg/ml) was taken as the limit of detection (LOD) for this HPLC method.

Recovery, within-day and inter-day precision

To obtain the extract recovery and accuracy of the two sample preparation methods for BKC in eye drops, spiked values (μg/ml) and measured values at 10.0, 20.0, and 50.0 μg/ml (n=5 for each concentration of BKC used) were compared. Precision was measured as the percent coefficient of variation over the concentration range from 5.0 to 50.0 μg/ml for BKC during the course of validation. Accuracy levels on a single analytical day (within-day) and on different days (inter-day) are shown in Tables 1~4.

Study on the stability of BKC

Freeze and thaw stability was tested by analyzing BKC spiked solutions undergoing three freeze (−20 °C)-thaw (room temperature) cycles on consecutive days. In this cycle, each BKC sample stored at −20 °C for 24 h was thawed completely, left for 2 h and then returned to freezing conditions for 24 h. This process was repeated for 5 d. On each day, the BKC content was determined using the HPLC method (Table 5) and described as the percentage of the amount of BKC analyzed on each day relative to the amount determined on the first day.

Preparation of azithromycin eye drops

Azithromycin was dispersed into a beaker containing about 1/3 of the final weight of water and stirred for 10 min at 100 r/min. 0.5 mol/L phosphoric acid was slowly added and stirred until the azithromycin dissolved completely. Then, the solution was adjusted to the desired pH with 0.5 mol/L phosphoric acid. BKC and sodium chloride were added to the solution and stirred for 10 min after each addition. The solution was brought to 1/2 of the final weight with water. In another 5 ml bottle, sodium hyaluronate was dispersed about to 1/2 of the final weight of water and stirred for 24 h until completely dissolved. The two parts were mixed after sterile filtration through a 0.22 μm millipore filter and then stirred for 10 min at 100 r/min. The mixture was aseptically dispensed into multi-dose containers and the BKC content was determined.

RESULTS AND DISCUSSION

Selectivity

A sharp peak of BKC was observed under the chromatographic conditions described in this paper. The retention time of BKC was determined as 10 min. No peaks interfered with the detection of BKC in the samples (Fig.2), indicating that the HPLC method is effective. A single peak for BKC was obtained using this HPLC method, unlike in the report on USP (Bravo, 2009). However, when we detected the BKC content according to the USP method, it still showed only one peak. This result may be related to the source of the BKC (Chen and Wang, 2005).

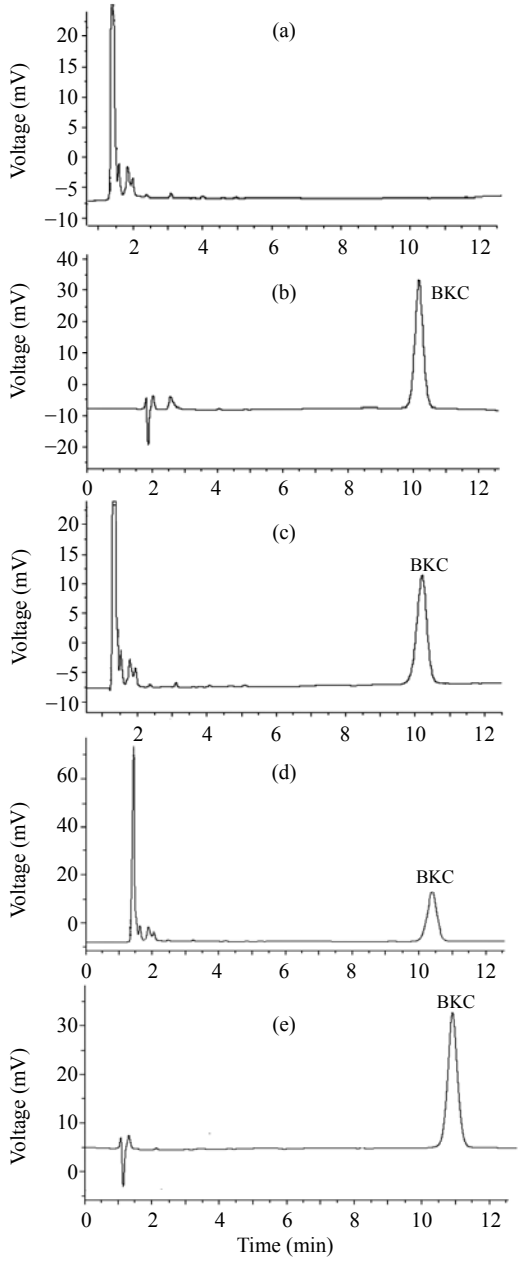

Fig.2 Representative chromatograms of BKC. (a) Control blank sample; (b) Control BKC at 30.0 μg/ml; (c) Standard with BKC 20.0 μg/ml; (d) Azithromycin eye drops containing BKC; (e) Control BKC at 30.0 μg/ml with USP method

Linearity

Good linearity between the concentration of BKC (C, μg/ml) in azithromycin eye drops and the average peak area (A) was obtained (C=3×10^{-5}A−0.954, r=0.9991, n=6) in the range from 5.0 to 50.0 μg/ml using deproteinization sample preparation. Using extract sample preparation, linearity was also good (C=2.83×10^{-5}A−0.849, r=0.9994, n=6). When the level was quantified over the range of the calibration curve, the sample was diluted with water to an appropriate concentration. This linearity was adequate for BKC determinations as low as 0.0005% (w/v) in dosage forms with viscous substances according to the sample preparation methods.

Limit of detection

The LOD (intra-day coefficient of variation (CV) ≤10%) for BKC in samples was found to be 0.10 μg/ml (S/N>3) as shown in Fig.3.

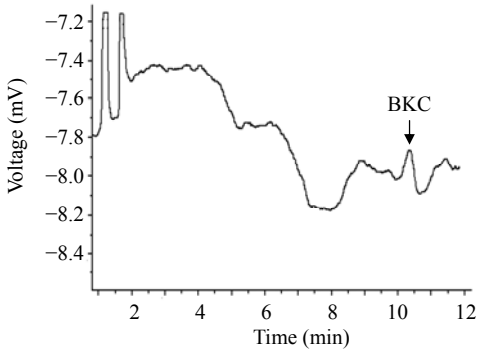

Fig.3 Limit of detection of BKC

Recovery, precision, and stability

Tables 1 and 3 list the recovery and precision data for BKC under the stated HPLC conditions using the deproteinization procedure. High recoveries were achieved at 10.0, 30.0, and 50.0 μg/ml. Table 3 shows both the within-day precision, with relative standard deviation (RSD) ranging from 0.63% to 1.13%, and the inter-day precision, with RSD ranging from 0.72% to 2.76%, indicating the very good reproducibility of

this method. Tables 2 and 4 list the recovery and precision data of BKC under the stated HPLC conditions using the extraction procedure. As shown above, recovery, precision and accuracy were adequate for BKC determination in dosage forms with viscous substances with these two sample preparation methods. But compared with the extraction procedure, the deproteinization procedure was much quicker and more convenient. So, the deproteinization procedure is a suitable sample preparation method which has benefits for BKC determination. Table 5 lists the content of BKC samples in freeze-thawing conditions. Over 5 d, the BKC content was stable, which means there was no degradation in the freeze and thaw cycle. The data indicated that the BKC samples had good stability in freeze-thawing conditions.

Table 1 Recovery of BKC with deproteinization procedure*

Added value (μg/ml)	Measured value (μg/ml)	Relative recovery (%)	RSD (%)
10.1	9.8±0.76	96.70	7.75
29.7	29.3±0.48	98.52	1.64
49.1	48.1±0.32	97.96	0.69

*Data are expressed as $\bar{\chi} \pm SD$ (n=5)

Table 2 Recovery of BKC with extraction procedure*

Added value (μg/ml)	Measured value (μg/ml)	Relative recovery (%)	RSD (%)
9.96	9.13±0.54	91.67	5.91
30.2	28.7±0.61	95.03	2.12
49.4	47.4±0.42	95.95	0.88

*Data are expressed as $\bar{\chi} \pm SD$ (n=5)

Table 3 Precision of BKC with deproteinization procedure*

Added value (μg/ml)	Within-day		Inter-day	
	Precision (μg/ml)	RSD (%)	Precision (μg/ml)	RSD (%)
10.2	9.96±0.064	0.63	9.71±0.073	0.72
30.1	29.6±0.34	1.13	29.4±0.83	2.76
49.7	49.3±0.58	1.12	48.8±1.07	2.15

*Data are expressed as $\bar{\chi} \pm SD$ (n=5)

Table 4 Precision of BKC with extraction procedure*

Added value (μg/ml)	Within-day		Inter-day	
	Precision (μg/ml)	RSD (%)	Precision (μg/ml)	RSD (%)
10.3	10.1±0.082	0.81	9.64±0.066	0.68
29.9	29.3±0.74	2.53	29.5±0.72	2.44
49.8	49.1±0.62	1.26	49.4±1.21	2.45

*Data are expressed as $\bar{\chi} \pm SD$ (n=5)

Table 5 Stability of BKC in freeze-thawing conditions

Time (d)	Content (%)	Time (d)	Content (%)
0	100.00	3	99.68
1	99.80	4	98.72
2	99.55	5	98.93

BKC content in azithromycin viscous ophthalmic drops

Batches of azithromycin viscous ophthalmic drops were prepared as described above and the BKC content was determined (Table 6). The average BKC content was about 0.0030% (w/w). This concentration is safe in the human body and this BKC concentration was used in AzaSite® eye drops developed by InSite Vision (USA) (Anonymity, 2008).

Table 6 BKC content in azithromycin viscous ophthalmic drops (n=6)

Batches	BKC content (%)	RSD (%)
1	0.0030	0.42
2	0.0028	0.36
3	0.0031	0.39

CONCLUSION

A simple and rapid HPLC method has been developed and validated for determination of the total BKC content in ophthalmic formulations containing viscosity excipients. The treatment of the sample is convenient, the precision and accuracy of the proposed method are within acceptable ranges, and the LOD is as low as 0.10 μg/ml. The LOD is low enough to detect the concentration of any other ophthalmic formulations involving viscosity excipients using an ultraviolet (UV) detector. In summary, this assay is a selective, sensitive, and reproducible method for the separation and determination of the total BKC content in ophthalmic formulations containing viscosity excipients.

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