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Short Communication

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Simple High Performance Liquid Chromatography Method for Determination of Benzalkonium Chloride Homologues in Nasal Preparations

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***SUMMARY*. A High-Performance Liquid Chromatography method was developed for quantification of benzalkonium chloride (BAC) and separation of three major homologues, in nasal preparations. Chromatography separation was carried out on a CLC-cyano column, using 0.075M acetate buffer with acetonitrile (45:55, v/v) pH = 5.0, as mobile phase, in an isocratic elution. The total run time was 20 min at a flow rate of 1.0 mL/min. The retention times were of 10.0, 12.8 and 16.1 min, respectively, for homologs C_{12}, C_{14} and C_{16} of benzalkonium chloride. About precision, the method presented excellent repeatability and satisfactory precision intermediate, and the coefficients of variation found were 1.73 and 4.51%, respectively. The accuracy values were ranging from 100.86 to 110.99%. The lower limits of quantification and detection were 5 µg/mL and 2.5 µg/mL, respectively. Two out of seven analyzed lots contained less BAC than allowed.**

INTRODUCTION

Benzalkonium chloride (BAC) is a bacteriostatic agent used in the pharmaceutical industry as active ingredient or a preservative. BAC, in the form of white or almost white crystals or gel, is a mixture of alkylbenzyldimethylammonium chlorides [1,2], being the three most important those with alkyl substituent C_{12}, C_{14}, C_{16} with the quaternary ammonium salt. In the literature, controversial information can be found concerning its toxic properties, which emphasized the quality control necessity. It was recently demonstrated [3-5] that BAC has a relevant cumulative toxic effect when administrated by the nasal route for a long period of time. However, Marple *et al.* [6], after performing a meta-analysis, concluded that BAC used as preservative in nasal preparations is safe and well tolerated for both short- and long-term use. Furthermore, BAC has antimicrobial potency and its influence on human health and the possible damage of human nose epithelia makes quantitative determination of its amount necessary.

BAC is a pharmacopeical substance, present in FP V, Ph. Eur., and USP XXIV. Only the USP monograph [7] specifies the percentage of each BAC homologue in relation to total BAC content. In FP V and Ph. Eur., titration, colorimetric and spectrophotometric methods, in which the total content of homologues is measured, is used for benzalkonium chloride determination [8]. It can be observed that pharmacopeical methods used in the analysis of pure BAC solutions are often insufficient to determine the compound in pharmaceutical preparations which contain various substances. In this case, sample preparation is complicated and the method is relatively unreliable.

Other described methods in literature has revealed that several methods such as capillary electrophoresis [9,10], gas chromatography-mass spectrometry [11], eletrokinetic chromatography [12]

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and high performance liquid chromatography [13] have been reported for the analysis of BAC either in pharmaceutical preparations [8,9,13,14] or in effluents [11,15,16].

Since the percentage of each homologue is crucial for BAC action, the correctly separation of C_{12}, C_{14} and C_{16} have to be done. Homologues differ in their physical, chemical and microbiological properties. The efficacy of a compound as a preservative depends on the content of appropriate homologues in the mixture [17]. Consequently, control of benzalkonium chloride identity and content in pharmaceutical preparations is necessary. In addition, the analytical method should permit the determination of both total BAC content and the content of each BAC homologue.

Thus, the aim of this work was to propose a rapid, simple and accurate method for benzalkonium chloride determination, in which their homologues were properly separated.

MATERIAL AND METHODS

Chemicals and reagents

Benzalkonium chloride reference standard [10% (p/ v), lot KOB 151] was purchased from the United Sates Pharmacopoeia, with specific gravity 0.9894. Acetonitrile (HPLC grade) and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Sodium acetate anhydrous was obtained from Sigma-Aldrich (St. Louis, USA). HPLC-water was obtained from a water purification system (Master System, GEHAKA, Brazil). Benzalkonium chloride content was determined in seven different lots of nasal pediatric preparations (liquid form), which contained benzalkonium chloride and sodium chloride.

Reference standard preparation

A standard USP grade BAC solution containing 2000 µg/mL diluted in the mobile phase was used as a standard throughout this work. BAC content is expressed as the sum of the three major homologues characterized using BAC USP standard. The concentration of BAC in the reference was selected so as to correspond to concentrations in a real sample of nasal preparation. Routine quality control was assessed by use of in-house BAC controls analyzed in each run.

Instrumentation

The HPLC system was a Shimadzu liquid chromatograph system consisting of a LC- 10AT vp pump; SCL-10A vp controller, SIL- 10AF vp autosampler; diode array SPD-10A vp detector and DGU - 14A degasser. The analytical column was a Shim-pack CLC-cyano (250 x 4.6 mm, internal diameter), 5 µm particle size, pore size of 100 Å - *Shimadzu*®). An injection loop of 25 µL was used. Data acquisition and quantification were performed using CLASS- Vp version 5.42 *software*.

Chromatographic conditions

In order to optimize the conditions of benzalkonium chloride separation occurring in the sample along with other substances, a number of experiments were performed, in which the influence of the solid phase (column type) and the mobile phase composition were evaluated.

As a result of the performed tests, the Shimadzu CLC-CN column (250 mm x 4.6 mm, particle size 5 µm, pore size 100 Å) and the following separation conditions were selected: 0.075M acetate buffer with acetonitrile (45:55, v/v) pH = 5.0, as mobile phase, in an isocratic elution. The flow rate was 1.0 mL/min, the column was kept at room temperature and the detection was made by a diode array detector (DAD) at a wavelength of 262 nm.

Different concentrations of benzalkonium chloride (USP standard) diluted in mobile phase were used to correlate the area and BAC content of samples. Tested samples, in the form of solutions, were injected directly on the column.

Method validation

The method was fully validated according to local guidelines (BRASIL, 2003). Specificity, linearity, precision, limit of detection (LOD), limit of quantification (LOQ), accuracy of the HPLC method and sample stability were determined.

Specificity

The specificity was evaluated by comparing the retention time of BAC homologues with those of naphazoline and the sodium chloride, the other component presented in nasal solution preparation.

Linearity

To evaluate the linear range of the present method, standard solutions were prepared in duplicate, and analyzed in three separate runs. The linearity (12.5 - 400 µg/mL) was calculated by the least square regression method.

Precision – Repeatability and Intermediate precision

Intra-day assays were performed using three replicates on three consecutive days and inter-day assays were performed on three separate days. The intra-day precision was estimated on the three concentrations (10, 25 and 100 µg/mL) and the inter-day precision on the same calibrations standard levels. The precision for BAC was not exceeded 5% at any of studied concentration, except for LOQ, which 15% is allowed.

Limits of detection (LOD) and quantification (LOQ)

The analytical response at LOQ can be detected with sufficient precision (15–20%) and accuracy (80–120%). LOD is defined as the lowest concentration of BAC at which the signal is three times larger than the one of the baseline.

Accuracy

The method accuracy was determined by directly analyzing BAC 100 and 25 µg/ mL concentrations. For each solution, three replicates were prepared and were injected three times. The accuracy was obtained calculating the percentage deviation between calculated concentrations from real concentrations. A bias of 15% is allowed.

Stability

Benzalkonium chloride stability was investigated in standard solutions at 200, 100 e 25 µg/mL concentrations. Stability of working solutions was evaluated under storage conditions: room temperature during 24 h and 7 days; 4 °C (24 h, 7 and 14 days); and –20 °C (24 h, 7, 14 and 28 days). A bias of 15% is allowed.

Samples quality control analyses

After developed and validated the method, seven different samples of nasal formulations were analyzed. Samples were homogenized and directly injected on the equipment. All samples contained 100 µg/mL benzalkonium chloride. This assay was carried out in triplicate.

RESULTS AND DISCUSSION

Under the described chromatographic conditions, the retention times of BAC homologues, C_{12}, C_{14}, C_{16}, were 10.0, 12.8 and 16.1 min, respectively. The retention time of naphazoline (50 µg/mL) was 5.2 min. No interfering peak was observed in the presence of sodium chloride. It can be visualized at Figure 1.

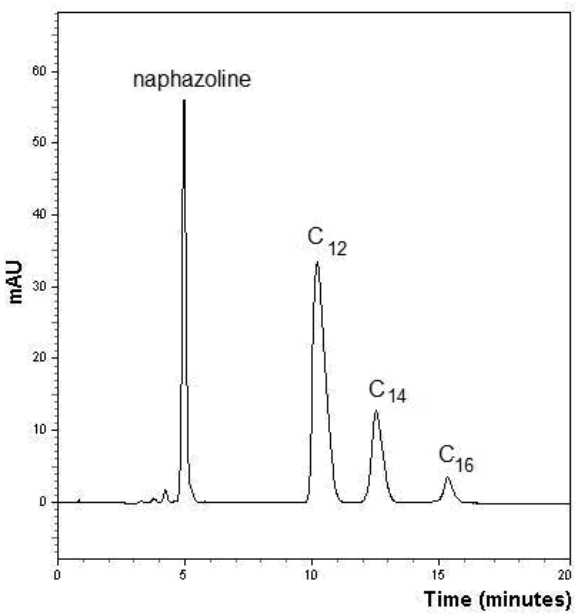

Figure 1. Chromatogram of benzalkonium chloride (BAC) standard (100 µg/mL) and naphazoline (50 µg/mL). Analytical conditions: Shimadzu CLC-CN column (250 mm x 4.6 mm, particle size 5 µm, pore size 100 Å); mobile phase (pH = 5.0): acetonitrile–0.075M acetate buffer [55:45 (v/v)], in an isocratic system; flow rate: 1.0 mL/min; UV-DAD detection at 262 nm; injection volume: 25 µL.

Quantitative determinations of BAC content in the selected preparations were performed. The determined content corresponded to the declared BAC content in the tested samples. The content was calculated from the sum of areas of the individual BAC homologues peaks present in a given preparation, compared to the sum of the same homologues in the standard. A linear dependence between the sum of areas under the homologues' peaks and benzalkonium chloride concentrations was observed. This relationship is represented by the following equation: $y = 66913\ x + 79132$ and correlation coefficient (R^2) = 1.0, which confirms the linear dependence in concentration range of 12.5-400 µg/mL. High separation between individual BAC homologues and the other components of the preparations indicates that the method has adequate selectivity and specificity.

The validated method had satisfactory precision both for intra-day and inter-day assays. Table 1 shows the results obtained for the intra-assay (variation intra-day) and inter-assay (variation inter-day) precision for BAC.

The limit of detection (LOD) was 2.50 µg/mL of BAC determined by progressive dilution

BAC concentration (µg/mL)	BAC concentration (CV %)			
	First day (n = 3)	Second day (n= 3)	Third day (n = 3)	Three days (n = 9)
100	0.26	0.07	0.21	0.20
25	0.87	0.56	0.72	0.72
10	1.73	1.37	1.08	1.64

Table 1. Precision (intra and inter-day), expressed as coefficient of variation (CV) of benzalkonium chloride (BAC) found in the USP standard.

method. The limit of quantification (LOQ) was 5 µg/mL with coefficient of variation (CV) of 2.5% and standard deviation (SD) of 0.02 µg/mL for three replicates. The method was considered accurate because the range found for the concentration was between 100.86 from 110.99%. All the stability experiments performed met the local guidelines requirements: the deviation from BAC initial concentrations was lower than 15% in the analyzed solutions. No significant loss of BAC (≤10%) was observed after storage of solutions: at room temperature for 24 h, which enables postal samples to be accepted for analysis; at 4 °C for 15 days and at –20°C for one month, which proves BAC stability under routine storage conditions.

Seven nasal formulation samples from pharmaceutical industries were analyzed. These samples belonged to seven different lots. Two out of seven lots contained less BAC than the expected amount (85-105 µg/mL). Results are shown in Table 2.

As a part of determining the conditions of the chromatographic analysis of BAC homologues, the influence of selected factors on the separation process were tested, particularly the impact of column packing type and the mobile phase composition were assessed.

In the available literature, columns with NH_2 functional groups [15], reverse phase columns [18,19] and columns with cyano packing [7,20,21] were used for BAC determination. In our preliminary assays, when a Waters, C18, 5 µm, 150 mm x 4.6 mm column and acetonitrile–0.075 M acetate buffer, pH = 5.0, in the proportion 55:45 (v/v), respectively, was used as the mobile phase, no peaks were obtained on the chromatogram. As a result of the performed tests, the Shimadzu CLC-CN column (250 mm x 4.6 mm, particle size 5 µm) and the mobile phase described, in an isocratic elution, flow rate was 1.0 mL/min and the column at room temperature, were used.

Samples	BAC concentration * (µg/mL)
1	88.06 ± 1.54
2	88.15 ± 5.16
3	84.58 ± 1.40
4	88.90 ± 0.83
5	88.93 ± 0.37
6	96.41 ± 0.79
7	79.63 ± 0.99

Table 2. Benzalkonium chloride (BAC) content of seven different samples of nasal formulations. *Results are expressed as concentration ± standard deviation (n = 3).

Bernal *et al.* [13] used in their study the HPLC method with a reverse phase column (150 mm x 4.6 mm Spherisorb 5 ODS-2), and acetonitrile with ultra-pure water (55:45), as mobile phase, in an isocratic elution, for concurrent determination of the active ingredient and BAC in Beconase and Flixonase. The detection was performed at 250 nm wavelength. Here, it was observed that it is not possible to determine BAC in the aforementioned conditions. For high concentrations of BAC the determination is possible, but the detection has to be performed at 210 nm. Fan *et al.* [22] and Kummerer *et al.* [15] determined BAC only after the sample has been extracted to the solid phase (SPE/HPLC). In our developed and validated method, this was not necessary. This fact represents a shorter time of analysis and lower costs.

According to the American Pharmacopoeia, columns with cyan packing are recommended for the separation of BAC homologues. Prince *et al.* [23] also used columns with cyan packing to separate BAC homologues in their study. Both the method described in the USP and the study of Prince *et al.* [23] concern the determination of a pure substance solution. Here, samples containing also sodium chloride and naphazoline as possible interferents.

In our study, when a separation of individual BAC homologues in pharmaceutical preparations was achieved using a cyano column, good

symmetric BAC peaks was obtained. The greater advantage of the presented method is no necessity of any previous treatment. Even a dilution was not done. The standard and tested sample peaks areas were comparable. The mixture described produced optimal separation with a total analysis time of 20 min. It was also observed that adjusting the detector wavelength at 262 nm, we obtained maximum sensitivity of BAC compared to that of 230 and 254 nm.

CONCLUSION

In conclusion, the developed method may be used for the assessment of identity and content of individual benzalkonium chloride homologues in various pharmaceutical preparations. The method is simple and does not require particular sample preparation for the tests. Comparing to the available applications described in the literature, this method is able to perform both a qualitative and quantitative assessment of individual homologues of the compound, and, as a consequence, to compare various substances declared as BAC. The method validated in this study is rapid, precise, accurate and specific for BAC quantification in nasal preparations.

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