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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 909–920
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Application of the HPLC method for benzalkonium chloride determination in aerosol preparations

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Received 14 June 2002; accepted 19 September 2003

Abstract

Benzalkonium chloride (BAC) is a bacteriostatic agent used in the pharmaceutical industry as a preservative. BAC is a mixture of alkylbenzyldimethylammonium chlorides, the three most important of which being those with alkyl substituents C12, C14, C16 at the quaternary ammonium salt.

The purpose of this study was to develop a method for determining benzalkonium chloride identity and content in aerosol preparations in which protein or steroid hormones are the active components. The high performance liquid chromatography (HPLC) method was used for this purpose. In the performed comparison of the influence of selected factors on the process of the separation of BAC homologues, a column with packing modified with cyan groups and mobile phase containing 0.075 M acetate buffer with acetonitrile (45:55), in an isocratic elution, was used for qualitative and quantitative determinations and for method validation. The developed method may be used for the assessment of the identity and content of BAC homologues in various pharmaceutical preparations. It is simple and it does not require particular sample preparation for the tests. It is characterized by good selectivity and high precision of the determinations.

Keywords: Benzalkonium chloride; Homologues BAC; HPLC method and validation

1. Introduction

Benzalkonium chloride (BAC) is a bacteriostatic agent used in the pharmaceutical industry, in particular in aerosol preparations, as a preservative. BAC, in the form of white or almost white crystals or gel, is a mixture of alkylbenzyldimethylammonium chlorides [1,2], the three most important of which being those with alkyl substituents C12, C14, C16 with the quaternary ammonium salt.

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 [3]. In view of the above, control of benzalkonium chloride identity and content in pharmaceutical preparations is necessary. In addition, the analytic method should enable to determine both total BAC content and the content of each BAC homologue.

BAC is a pharmacopoeial substance, present in FP V, Ph. Eur., and USP XXIV. Only the USP monograph [4] specifies the percentage of each BAC homologue in relation to total BAC content. In FP V and Ph.

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doi:10.1016/j.jpba.2003.09.001

Eur., titration, colorimetric and spectrophotometric methods, in which the total content of homologues is measured, are used for benzalkonium chloride determination. Therefore, at the 103rd Session of the European Pharmacopoeia Committee, a proposal of supplying the monograph with preparation composition testing was put forward. Currently, a fixed value of the substance molecular weight is given, although in practice it is a variable depending on the preparation composition [5]. Pharmacopoeial 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, preparing a sample preparation is complicated and the method is relatively unreliable. Recently, the HPLC method has been often used in the analysis of pharmaceutical preparations.

The purpose of this study was to develop a method for determining benzalkonium chloride identity and content in aerosol preparations in which protein or steroid hormones, or their analogues, are the active components. The developed method should be characterized by an uncomplicated sample preparation technique as well as by high specificity and sensitivity. It should be fast and applicable in a wide range of situations.

High performance liquid chromatography (HPLC) method was used for this purpose.

2. Experimental

2.1. Equipment

Shimadzu liquid chromatograph equipped with two LC-10AT vp pumps, SCL-10A vp controller connected to a computer (Class-VP 5.03), SIL-10AD vp autosampler, UV-Vis SPD-10A vp detector and a CTO-10AC vp column oven.

2.2. Materials and reagents

Acetonitrile (Lab-Scan), sodium acetate trihydrate (Promochem), glacial acetic acid (AppliChem) and water were used in the tests. Acetonitrile and water purity grades were compliant with the requirements of the HPLC method; the other reagents were analytically pure.

USP benzalkonium chloride reference standard was used—catalogue number 5100, specific gravity 0.9894.

Benzalkonium chloride content was determined in the following preparations: Miacalcic Nasal 200 (Novartis), Synarel (Searle, Division of Monsanto p.l.c.), Flixonase (GlaxoWellcome) and Nasonex (Schering-Plough). All these preparations have the form of aerosols. Miacalcic and Synarel contain protein hormones: calcitonin and nafarelin, respectively. The preparations Flixonase and Nasonex contain glycocorticosteroids: fluticason propionate and mometazone furoate, respectively. The tested preparations contained benzalkonium chloride as a preservative.

2.3. Method

High performance liquid chromatography method was used to determine benzalkonium chloride homologues in various pharmaceutical preparations.

In order to optimise 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 (ionic strength of the buffer and phase composition), and the column temperature, were measured. The tested samples were prepared depending on preparation composition. The effect of the sample pH on the achieved separations was also evaluated.

As a result of the performed tests, the Phenomenex column (type Luna 5 μm CN, 250 mm × 4.6 mm) and the following separation conditions were selected: mobile phase acetonitrile–0.075 M acetate buffer, pH 5.0, in the proportion 55:45 (v/v), respectively, in an isocratic system. Flow rate was 1 ml/min., and the column temperature −25 °C. The detection was performed at 262 nm wave length, and the injection volume was 50 μl.

As a standard, 0.1 mg/ml benzalkonium chloride water solution (USP Standard) was used. Tested samples: Miacalcic and Synarel preparations, in the form of solutions, were injected directly on the column. The glycocorticosteroid preparations, in the form of suspensions, contain a number of excipients, such as microcrystalline cellulose, sodium carboxymethyl cellulose, phenylethyl alcohol and polisorbat 80, apart from the active components. In this case, the tested

sample was prepared as follows: 10 ml acetonitrile was added to 1 g Flixonase or Nasonex, and the sample was stirred with a mechanical stirrer for 1 min. Then the sample was centrifuged for 5 min (3200G). The obtained supernatant was injected on the column.

2.4. Method validation

Method validation was performed by determining: specificity, linearity, precision and accuracy, and the limits of determinability. Method resistance and versatility were also tested.

3. Results and discussion

As part of determining the conditions of the chromatographic analysis of BAC homologues, the influence of selected factors on the separation process were tested. The impact of column packing type, mobile phase composition, buffer ionic strength and column temperature was assessed.

In further analyses, a comparison of the following parameters was performed: retention time, separability, number of theoretical plates, provided that the areas of the individual homologues peaks should not differ significantly.

In the available literature, columns with NH_2 functional groups [6], reverse phase columns [7,8] and columns with cyan packing [4,9,10,11] were used for BAC determination. In our study, when a Kromasil 5, 100 Å, NH_2, 5 μm, 250 mm × 4.6 mm column and acetate buffer with acetonitrile were used as the mobile phase, no peaks were obtained on the chromatogram. Bernal et al. [12] in their study used the HPLC method with a reverse phase column (150 mm × 4.6 mm Spherisorb 5 ODS-2), and acetonitrile–water, 55:45, as the mobile phase, for concurrent determination of the active ingredient and BAC in Beconase and Flixonase. The detection was performed at 250 nm wave length. It was observed that it is not possible to determine BAC in the above separation conditions. For high concentrations of BAC (10× higher than those in the preparations) the determination is possible, but the detection has to be performed at 210 nm wavelength. Fan et al. [13] and Kummerer et al. [6] determined BAC only after the sample has been extracted to the solid phase (SPE/HPLC). The chromatogram presented in Fig. 1 shows the results for standard and tested samples (Miacalcic Nasal), registered in our study with a Luna C8, 5 μm, 250 mm × 4.6 mm column. One large asymmetric benzalkonium chloride peak (23 min retention time) was obtained. The areas of standard and tested sample peaks were comparable. Thus, it has been shown that quantitative BAC determination is possible on a C8 column, but there is no possibility to separate individual homologues. According to the American pharmacopoeia, columns with cyan packing are recommended for the separation of BAC homologues. Prince et al. [10] 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. concern the determination of a pure substance solution. In our study, a separation of individual BAC homologues in compound pharmaceutical preparations was achieved using a Luna CN, 5 μm, 250 mm × 4.6 mm column. Separation parameters are presented in Table 1.

In order to optimise the method, the effect of buffer ionic strength (0.075 M and 0.15 M), acetonitrile content in the mobile phase and the column temperature on the separation of BAC homologues was assessed. It has been shown that the above factors influence the retention times and the separability of BAC homologues. The obtained results are presented in Figs. 2–4.

The influence of sample pH on the quality of the separation of BAC homologues was assessed. The analyses were performed for standard solutions of pH 6.42; 5.11; 2.92 (concentration range observed in the pharmaceutical preparations selected for analysis). No influence of pH on the obtained separations was found, which is demonstrated in Fig. 5.

In the performed comparison of the selected factors' influence on the process of the separation of BAC

Table 1
Parameter of separation of USP standard BAC

Parameter	Homologues of BAC			
	C10	C12	C14	C16
Retention time (min)	14.642	16.575	19.150	21.958
Peak area	198	184873	60618	11056
Resolution	–	2.89	3.90	4.02
Theoretical plates	–	8970	12701	12955

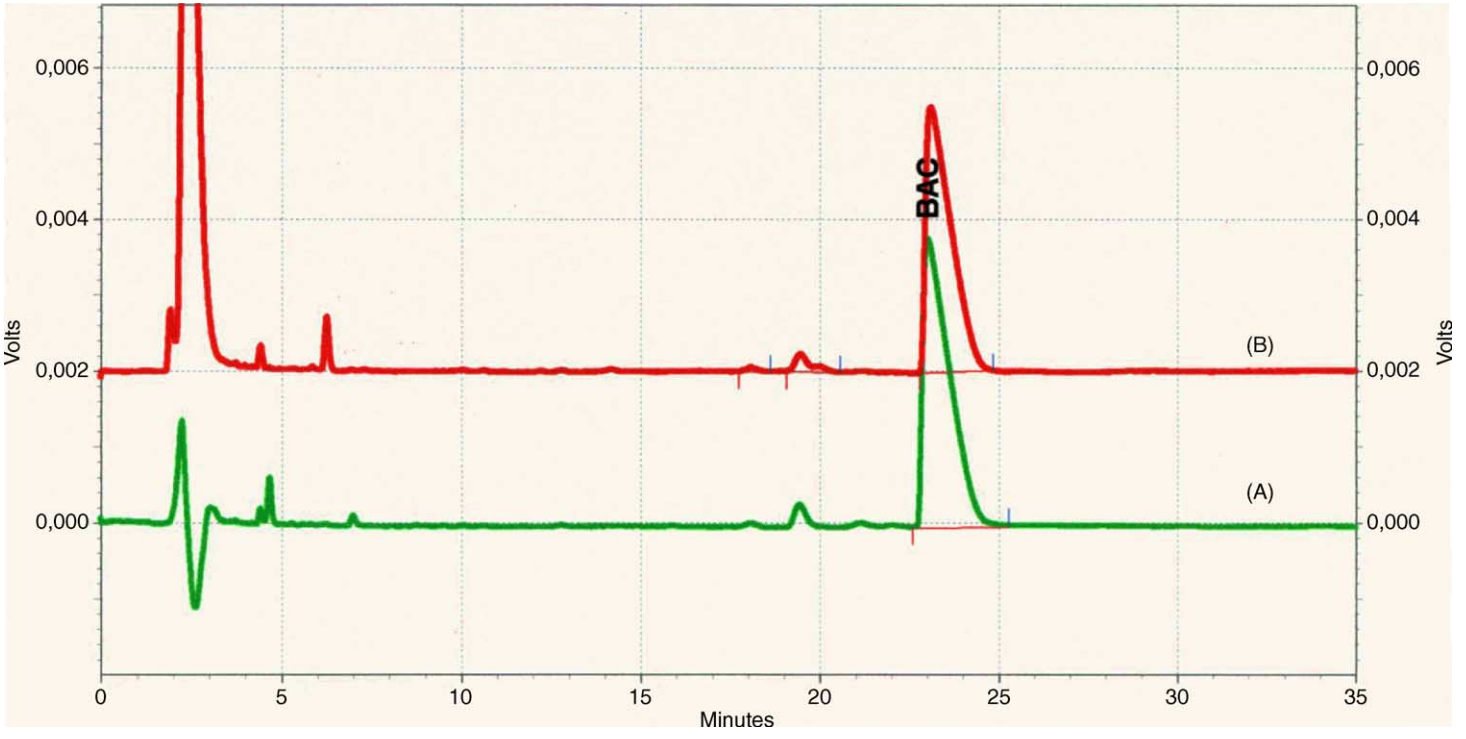

Fig. 1. Chromatograms of: (A) BAC-standard and (B) Miacalcic Nasal 200; column: Luna C8, 5 μm, 250 mm × 4.6 mm, mobile phase: acetonitrile–buffer acetate (0.075 M) (55:45, v/v). Commentary: Only one peak for BAC obtained in these conditions. The separation of homologues has not achieved.

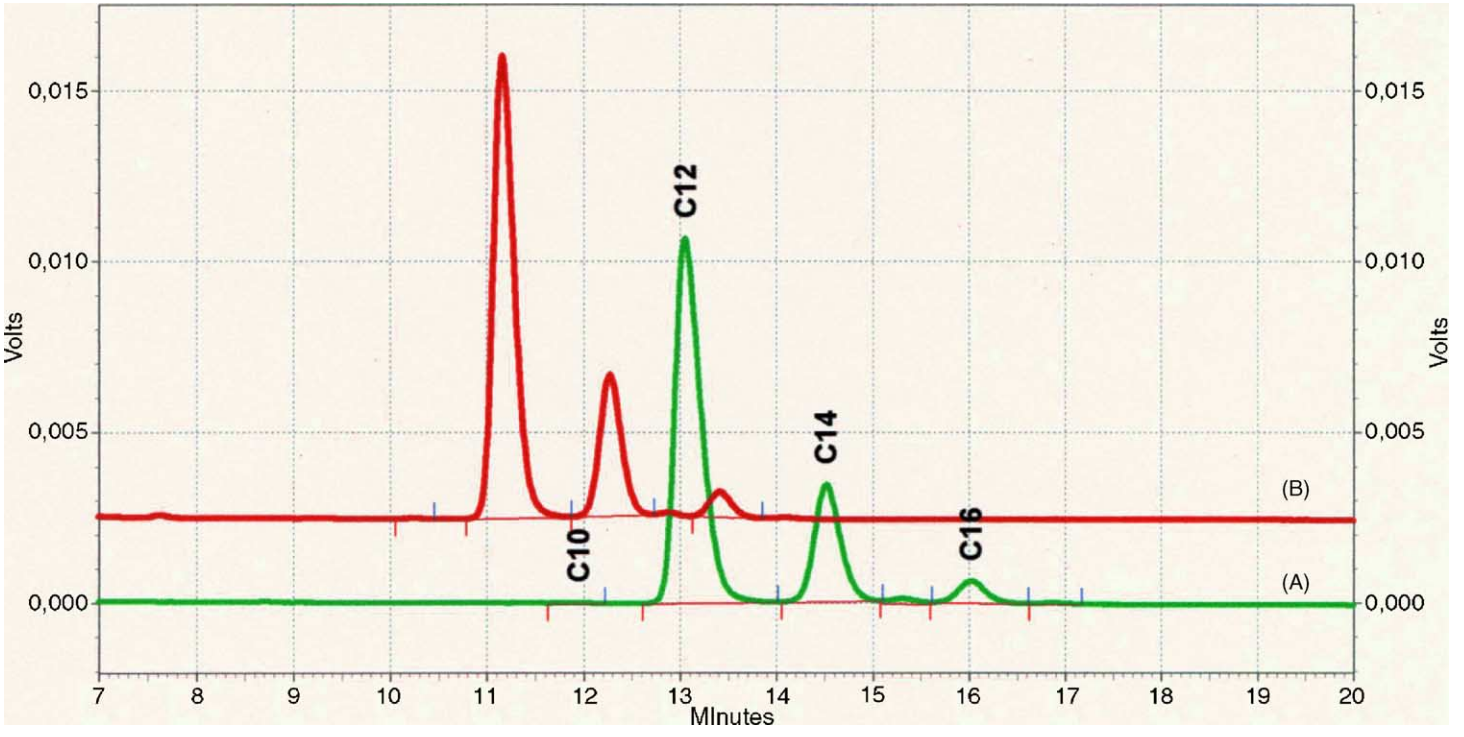

Fig. 2. Chromatograms of BAC-standard. Influence of ionic strength acetate buffer in a mobile phase: (A) acetonitrile–acetate buffer (0.075 M) (60:40, v/v) and (B) acetonitrile–acetate buffer (0.15 M) (60:40, v/v). An ionic strength acetate buffer changes only retention time of homologues BAC.

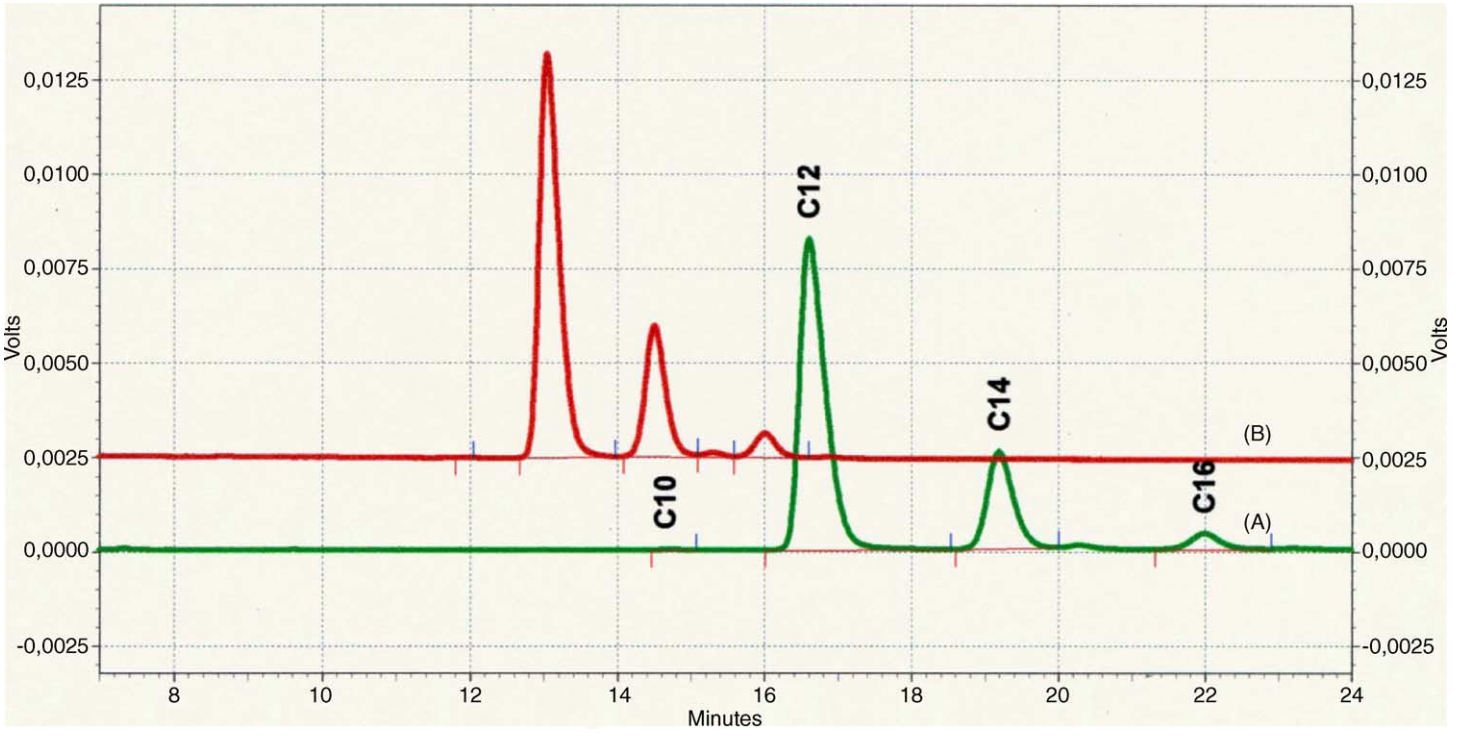

Fig. 3. Chromatograms of BAC-standard present an influence of content acetonitrile in a mobile phase: (A) acetonitrile–acetate buffer (0.075 M) (55:45, v/v) and (B) acetonitrile–acetate buffer (0.075 M) (60:40, v/v). A content acetonitrile in a mobile phase changes only retention time of homologues BAC.

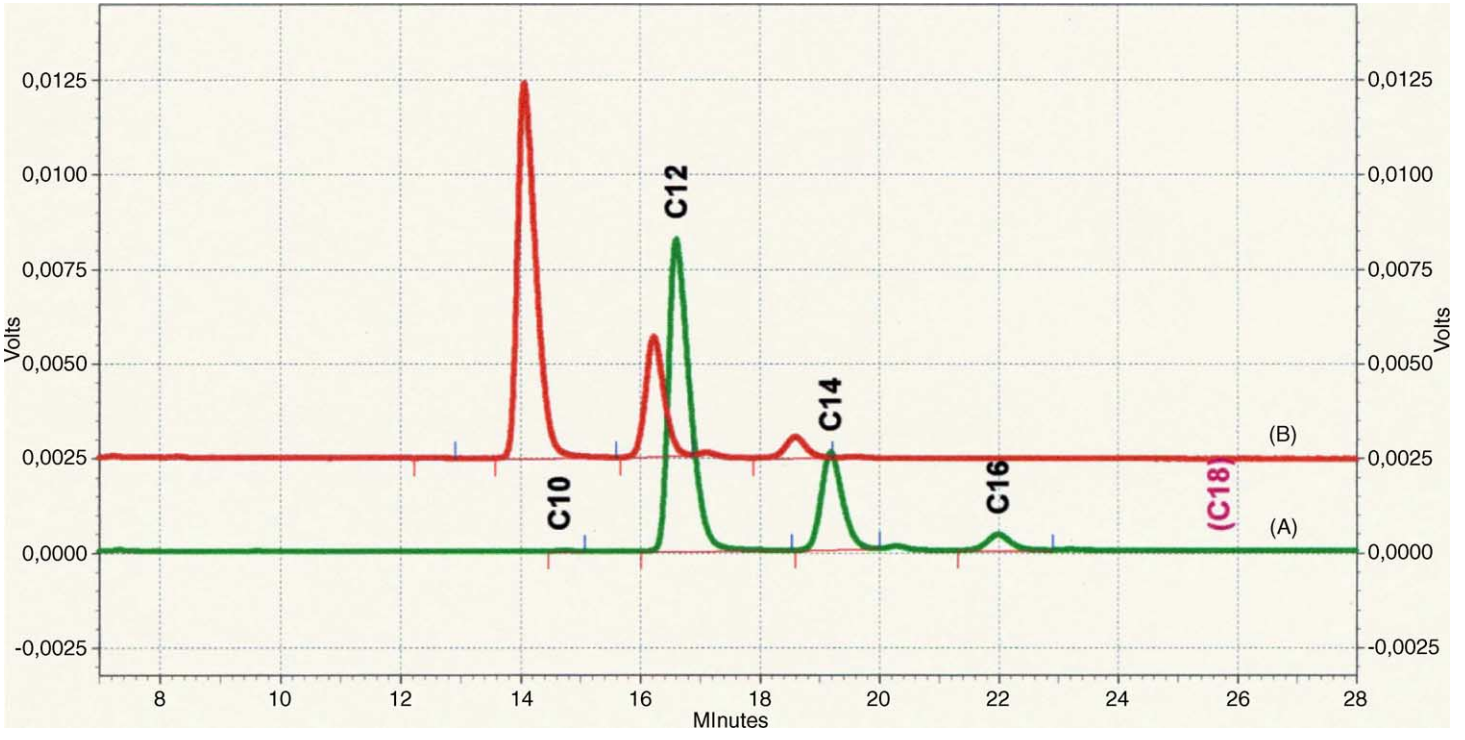

Fig. 4. Chromatograms of BAC-standard. Influence of column's temperature: (A) 25 °C and (B) 35 °C. A column's temperature changes only retention time of homologues BAC.

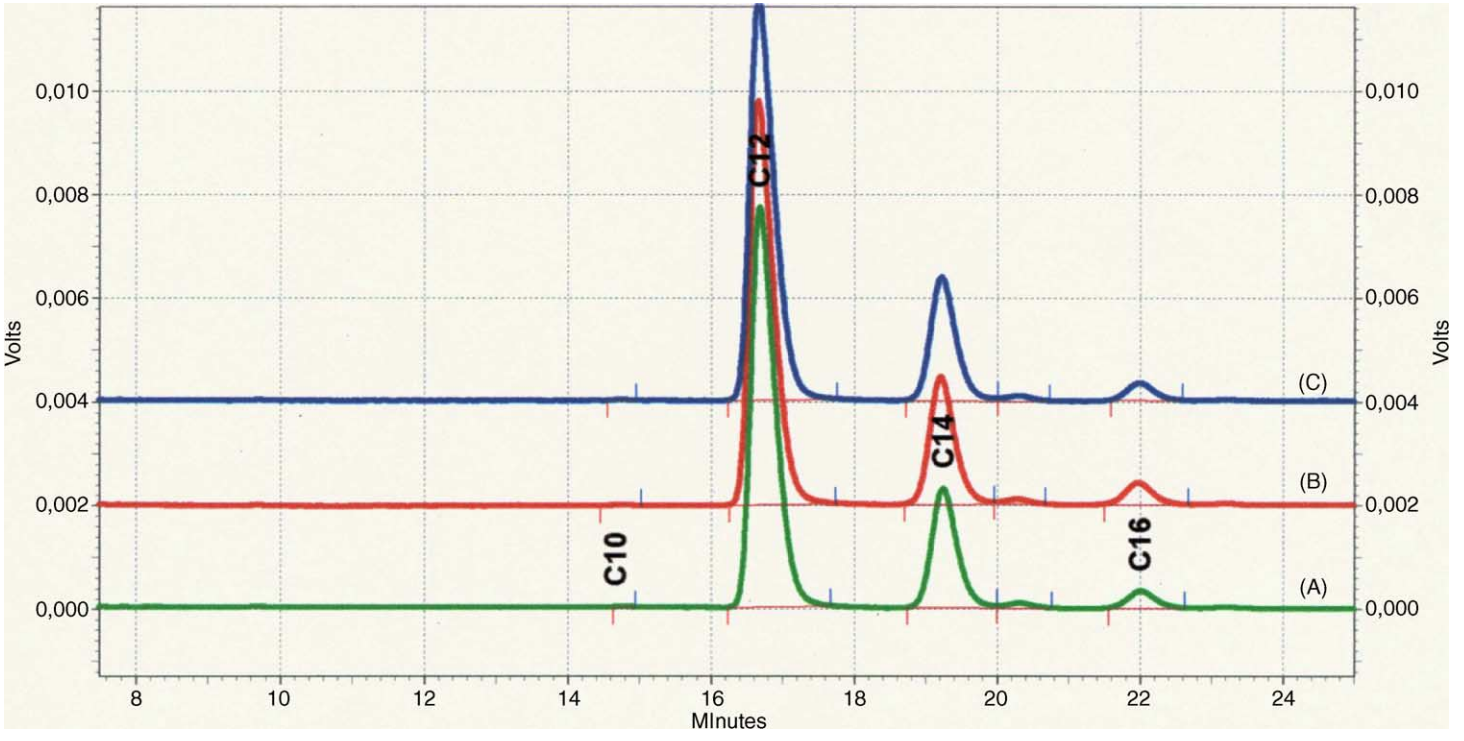

Fig. 5. Chromatograms of BAC-standard solution at different pH of samples: (A) pH 6.42; (B) pH 5.11; (C) pH 2.92. pH of samples does not influence on the separation of homologues BAC.

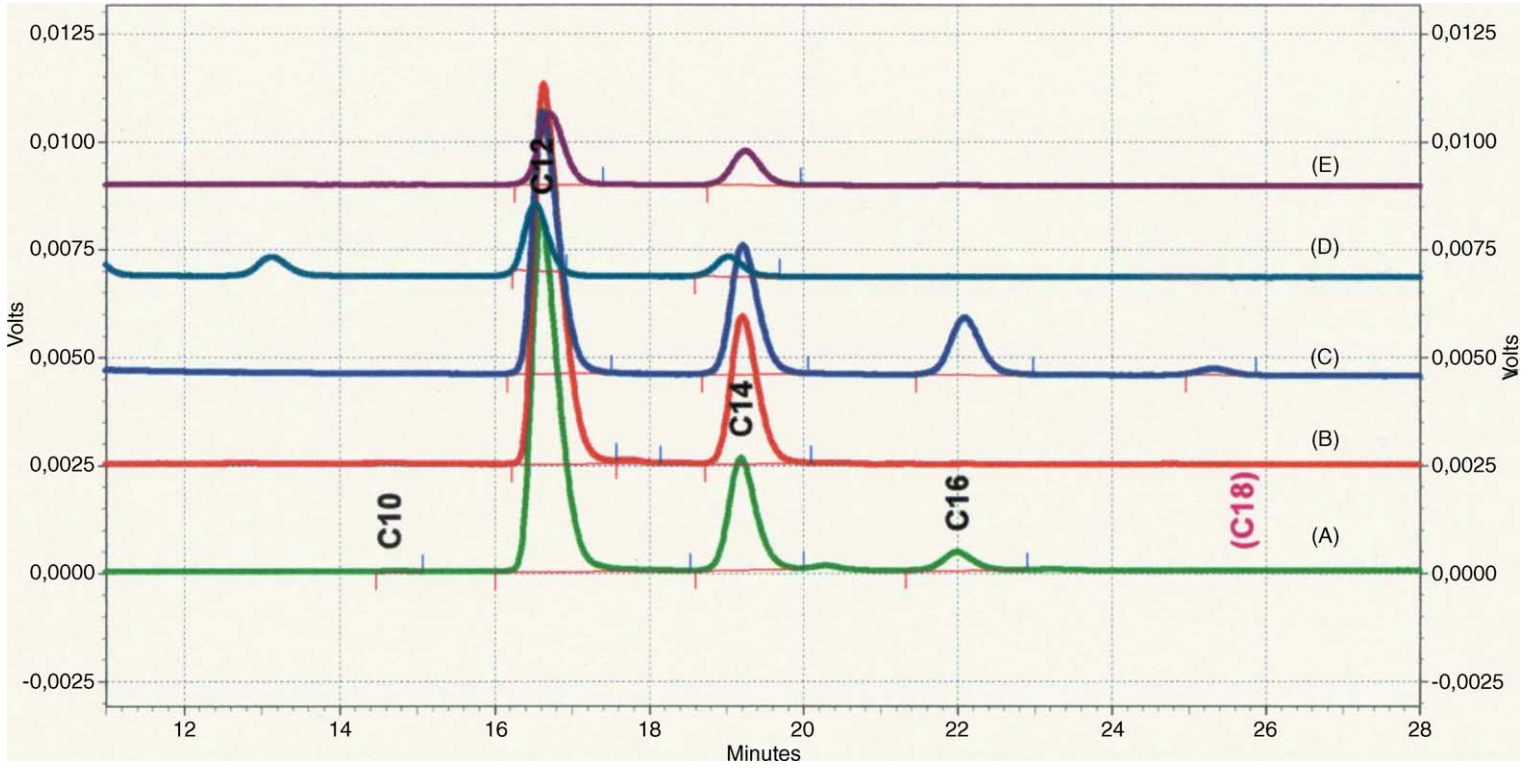

Fig. 6. Representative chromatograms of: (A) BAC-standard; (B) Miacalcic Nasal 200; (C) Synarel; (D) Flixonase; (E) Nasonex; UV detection at 262 nm, mobile phase: acetonitrile–acetate buffer (0.075 M) (55:45, v/v). Resolution of all homologues BAC in samples.

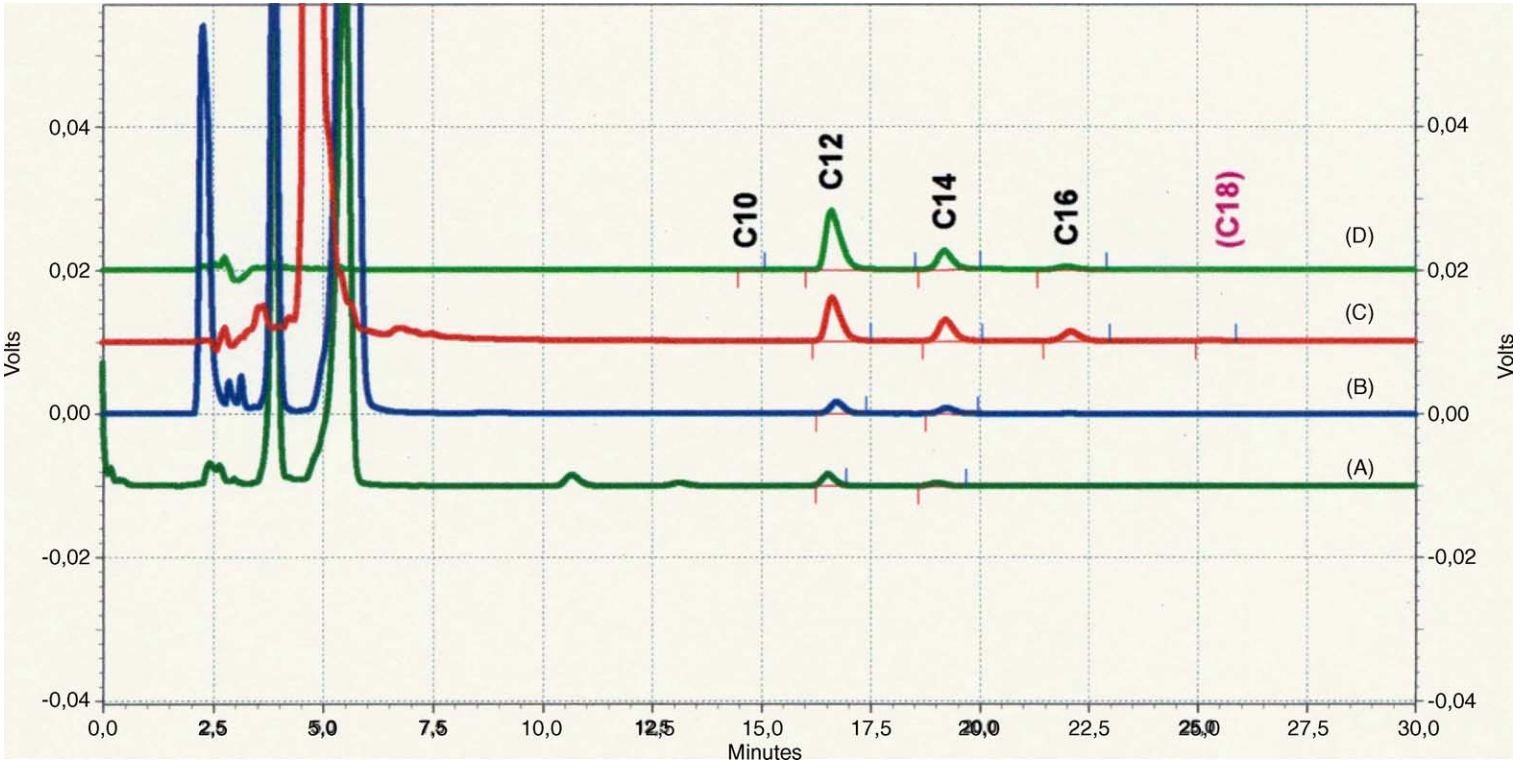

Fig. 7. Chromatograms of: (A) Flixonase; (B) Nasonex; (C) Synarel; (D) BAC-standard. Separation of homologues BAC from other compounds in samples. Specificity of method.

Table 2
Assay of benzalkonium chloride in pharmaceutical preparations

Name of preparation	Declared value (mg/ml)	*n*	Content of homologues (%)					Total peaks area	Result of determination (mg/ml)
			C10	C12	C14	C16	C18		
USP standard benzalkonium chloride	0.1	5	0.1	67	26	7	–	254404	–
Miacalcic Nasal 200	0.1	7	–	72	28	–	–	253195	0.0995 ± 0.0001
Synarel	0.1	7	–	55	29	14	2	259994	0.1022 ± 0.0002
Nasonex	0.2	7	–	65	35	–	–	596320	0.2344 ± 0.0009
Flixonase	0.2	7	–	77	23	–	–	498220	0.1958 ± 0.0008

homologues, a column with packing modified with cyan groups and a mobile phase containing 0.075 M acetate buffer with acetonitrile (45:55), in an isocratic elution, was used for qualitative and quantitative determinations and for method validation. The selected chromatographic system allows for the separation of individual BAC homologues from the other compounds, including the active ingredient (protein or steroid) and excipients, without a complicated sample preparation phase.

The developed method allows fast assessment of benzalkonium chloride identity, as its homologues migrate between the 14th and the 26th minute (Fig. 6). Identical retention times of the BAC standard (water solution) peaks and the BAC peaks in the preparations show that the active ingredient and the excipients present in the preparation have no effect on the retention times of the homologues (Fig. 7). High separability between individual BAC homologues and the other components of the preparations indicates that the method has adequate selectivity and specificity.

Quantitative determinations of benzalkonium chloride 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. The obtained results are shown in Table 2.

A validation of the HPLC method, discussed in the study, was performed. Linearity, repeatability and accuracy were assessed.

A linear dependence between the sum of areas under the homologues' peaks and benzalkonium chloride concentrations was shown. This relationship is represented by the following equation: $y = 2934950.581x - 13937.928$, with the correlation coefficient of 0.997, which confirms the linear dependence in concentration range of 0.0125–0.35 mg/ml.

Method precision was assessed by determining its repeatability and accuracy. Method repeatability was determined for the preparations specified in Table 3, by performing seven repeated measurements. The obtained variation coefficients indicate that the used chromatographic separation conditions are appropriate for benzalkonium chloride content determination in the analysed preparations.

Method accuracy was assessed for all tested preparations. The obtained results are shown in Table 4. The tests were performed for samples containing

Table 3
Precision of the method (repeatability)

Sample	*n*	Summed peak areas of BAC	S.D.	R.S.D. (%)	Confidence limit
Miacalcic Nasal 200	7	253195	222	0.10	$253189.7 < X < 253200.3$
Synarel	7	259993	709	0.27	$259976.2 < X < 260009.8$
Nasonex	7	59706	228	0.38	$59700.6 < X < 59711.4$
Flixonase	7	49828	211	0.42	$49823.0 < X < 49833.0$

Table 4
Precision of the method (accuracy) determination in Miacalcic Nasal 200, Synarel, Flixonase, Nasonex

Sample	n	Determination (%)	Declared value (mg/ml)	Value found (mg/ml)	Recovery (%)	Statistical evaluation
Miacalcic Nasal 200	3	80	0.06988	0.06933	99.21	$X = 99.20$; $n = 9$; S.D. = 0.431; %R.S.D. = 0.434; 99.19% < X < 99.21%
	3	100	0.08735	0.08661	99.15	
	3	120	0.10488	0.10407	99.23	
Synarel	3	80	0.08012	0.07970	99.48	$X = 99.66$; $n = 9$; S.D. = 0.251; %R.S.D. = 0.252; 99.65% < X < 99.67%
	3	100	0.10015	0.09983	99.68	
	3	120	0.12018	0.11997	99.82	
Flixonase	3	80	0.01525	0.01529	100.25	$X = 101.40$; $n = 9$; S.D. = 1.02; %R.S.D. = 1.00; 101.38% < X < 101.42%
	3	100	0.01906	0.01942	101.90	
	3	120	0.02287	0.02335	102.07	
Nasonex	3	80	0.01826	0.01821	99.68	$X = 100.77$; $n = 9$; S.D. = 1.13; %R.S.D. = 1.12; 100.75% < X < 100.79%
	3	100	0.02283	0.02302	100.82	
	3	120	0.02740	0.02789	101.80	

80, 100 and 120% of the declared value in individual preparations. Mean recovery percentage was approximately 99.5% for protein preparations and approximately 101% for steroid preparations. In all measurements, very low variation coefficients were obtained (the highest for Nasonex—1.12%).

4. Conclusions

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 allows performing a qualitative and quantitative assessment of individual homologues of the compound, and in consequence, to compare various substances declared as BAC. It is characterised by high selectivity and high measurement precision. The performed validation confirmed the usefulness of the method.

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