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Determination of benzalkonium chloride in aqueous ophthalmic preparations by high-performance liquid chromatography*

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Abstract: A high-performance liquid chromatographic method is described for the determination of homologues of benzalkonium chloride in aqueous ophthalmic preparations. The technique involves direct injection of the sample on a 5-μm Spherisorb-CN column. The mobile phase is acetonitrile–triethylamine (0.1%, v/v) in water (pH 2.5; 40:60, v/v). Detection is carried out at 215 nm. The method is rapid, specific, reproducible and simple, and is especially useful for the assay of this preservative in stability studies and quality control procedures.

Keywords: *Benzalkonium chloride; ophthalmic preservatives; benzalkonium homologues; cyano-propyl column; triethylamine-eluent modifier; HPLC.*

Introduction

Benzalkonium chloride (BAK) is a mixture of *n*-alkylbenzyl-dimethylammonium chlorides, with *n*-alkyl chain lengths of C_8H_{17} to $C_{18}H_{37}$. This mixture has a bactericidal effect against Gram-positive and Gram-negative bacteria, and is widely used as an antimicrobial preservative in topical aqueous pharmaceutical preparations, especially in ophthalmic solutions.

The homologues do not possess identical bactericidal activity [1], and, in order to ensure the efficacy of BAK, the USP [2] requires a minimum percentage of the main derivatives ($C_{12}H_{25}$ and $C_{14}H_{29}$) in the mixture. This means that it is necessary to determine the total content of BAK and also the proportions of its homologues in formulations that contain this preservative.

For eye drops, such determinations are not easy because BAK is present in low concentrations (40–100 ppm), whereas other ingredients are present in concentrations as high as 10%. High-performance liquid chromatography (HPLC) with UV detection is a suitable technique because of its high separation capability and high sensitivity at low wavelengths.

HPLC methods have been described for the determination of BAK homologues in raw materials, by means of a porous–polymeric HP-01 column [3] or a cyano-propyl-silica bonded stationary phase [4]. The second approach, with a previous ion-pairing extraction, has been applied to the assay of BAK in ophthalmic solutions [5]. Another method of determining this preservative in eye drops provides good results in samples containing slightly water-soluble products [6].

This paper describes an HPLC method that allows the determination of BAK homologues in the presence of a wide variety of common components of aqueous ophthalmic formulations. Neither extraction nor dilution is needed in solutions; centrifugation of suspensions is the only handling required. Separation is carried out by means of a Spherisorb-CN stationary phase with acetonitrile–aqueous triethylamine at an acidic pH as the mobile phase. Detection is at 215 nm, at which wavelength BAK has a high absorptivity; thus the assay can be carried out at ppm levels with good accuracy.

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Experimental

Solvents and chemicals

Benzalkonium chloride USP Reference Standard (batch I) was supplied by USPC Inc. (Rockville, USA). The composition of homologues was: C_{10} = 0.8%, C_{12} = 41.5%, C_{14} = 49.0%, C_{16} = 8.7% (w/w). Active ingredients and excipients were all USP and/or EP grade. Triethylamine (TEA), phosphoric acid (analytical grade) and acetonitrile (HPLC grade) were supplied by Scharlau S.A. (Barcelona, Spain). Water was purified by means of a Milli-Q water system, from Millipore (Barcelona, Spain). The 125 × 4.6 mm i.d. column was filled with 5-μm Spherisorb-CN (cyanopropyl-bonded phase), from Teknokroma (Barcelona, Spain).

Equipment

A Hewlett–Packard HP 1090M liquid chromatograph with automatic injector, thermostatic oven and diode-array UV detector was used. To print, integrate and compare the obtained chromatograms, an HP 79994A ChemStation was used.

Standard and sample solutions

Test solutions of BAK, excipients (21 different products listed in Table 1) and active ingredients (26 different products summarized in Table 2) were prepared with purified water.

No preparation was needed for samples of aqueous solutions. Before injection, aqueous suspensions were centrifuged until a clear solution was obtained.

Chromatographic conditions

The column was kept at 40 ± 1°C; the injection volume was 10 μl and elution was performed at a flow rate of 2.0 ml min^{-1}. The absorbance was monitored at 215 nm, at a bandwidth of 4 nm and a threshold of 1.0 mAU. The mobile phase was acetonitrile–TEA (0.1%, v/v) in water (pH adjusted with phosphoric acid to 2.5; 40:60, v/v).

Results and Discussion

Influence of mobile phase composition on selectivity

The effect of the mobile phase composition on the interaction of BAK with the cyanopropyl stationary phase was tested. Three parameters of the mobile phase were considered to have the strongest influence on the chromatographic separation: proportion of acetonitrile; concentration of TEA; and pH. The effects of these factors were examined in the range of conditions where they provided acceptable retention and resolution of BAK.

Three types of interaction of BAK with this stationary phase [7] may occur. One is a hydrophobic interaction between the cyanopropyl group and the alkyl chains of BAK. Second, a hydrogen-bonding interaction may occur between the free silanols of the silica support and the BAK–ammonium group; this interaction has a negative effect, causing broad, asymmetric and late-eluting peaks. Finally, a surface-ion interaction can take place between the nitrile of the stationary phase and the BAK–ammonium group.

Effect of proportion of acetonitrile. This effect was tested at proportions of 30–45% (v/v) of acetonitrile. A negative and linear relation (Fig. 1) was observed between the retention and resolution of the homologues and the proportion of acetonitrile. The hydrophobic interaction between BAK and the stationary phase decreased with a rise in the proportion of the organic solvent.

Effect of TEA. This effect was tested at TEA concentrations of 0.0–0.5% (v/v) in the aqueous fraction. TEA was shown to be the parameter with the major effect in the interaction of BAK with the stationary phase (Fig. 2). Its addition at a low concentration to the mobile phase reduced dramatically the retention times of BAK homologues, narrows the peaks, improved the sensitivity and slightly enhanced the resolution. For instance, when a mobile phase of acetonitrile–water (pH 2.5; 40:60, v/v) was changed to acetonitrile–TEA (0.1%, v/v) in water (pH 2.5; 40:60, v/v) the retention of the homologue C_{12} decreased about four times (K' from 30.7 to 8.6) while the resolution of the C_{12} homologue from the C_{14} homologue increased slightly (Rs from 1.6 to 2.4). However, the use of higher concentrations of TEA had a negative effect, causing low retention (for a constant acetonitrile proportion).

The effect of the addition of TEA to the mobile phase can be explained because it acts as a free silanol blocking agent [7]; however, it also competes with the ammonium group of BAK for the surface-ion interaction with the

Table 1
Selectivity of the method in the presence of common excipients in eye drops

Product	Concentration* (mg ml^{-1})	Result
Dextran 70	10	No interference
Glycerin	10	No interference
Hydroxyethylcellulose (Natrosol)	5	No interference
Hydroxypropylmethylcellulose (Methocel E4M Premium)	5	No interference
Mannitol	19	No interference
Methylcellulose (Thylose)	5	No interference
Polyethylene glycol 1540	30	No interference
Polysorbate 80 (Tween 80)	5	No interference
Polyvinyl alcohol (Airvol 165)	14	Interference†
Polyvinyl pyrrolidone	35	No interference
Propylene glycol	10	No interference
Sodium acetate	4	No interference
Sodium bicarbonate	1	No interference
Sodium bisulphite	3	No interference
Sodium borate	30	No interference
Sodium citrate	0.5	No interference
Sodium chloride	9	No interference
Sodium edetate	1	No interference
Sodium phosphate	30	No interference
Sodium thiosulphate	2	No interference
Sorbitol	10	No interference

*Usual concentration in eye drops.
†Insoluble in the mobile phase. Before analysis the sample should be diluted (1/5) in the mobile phase in order to precipitate PVA. The obtained supernatant can be injected without interference (injection volume = 50 µl).

Table 2
Selectivity of the method in the presence of common active ingredients in eye drops

Product	Concentration* (mg ml^{-1})	Result†
Antazoline phosphate	5	No interference
Atropine sulphate	10	No interference
Chloramphenicol succinate	20	No interference
Chromocarb diethylamine	100	No interference
Cromolyn sodium (sodium cromoglycate)	40	Interference†
Cyclopentolate hydrochloride	10	No interference
Dexamethasone sodium phosphate	1	No interference
Dipivefrin hydrochloride	1	No interference
Fluorescein sodium	20	Interference†
Gentamicin sulphate	6	No interference
Homatropine hydrobromide	10	No interference
Idoxuridine	2	No interference
Naphazoline hydrochloride	0.5	No interference
Neomycin sulphate	4	No interference
Oxybuprocaine hydrochloride	4	No interference
Phenylephrine hydrochloride	100	No interference
Pilocarpine hydrochloride	50	No interference
Polymyxin B sulphate	1	No interference
Scopolamine hydrobromide (hyoscine hydrobromide)	2.5	No interference
Sulphacetamide sodium	100	No interference
Tetracaine hydrochloride (amethocaine hydrochloride)	10	No interference
Tetrahydrozoline hydrochloride	0.5	No interference
Timolol maleate	5	No interference
Trifluridine	10	No interference
Trimethoprim	1	No interference
Tropicamide	10	No interference

*Usual concentration in eye drops.
†Eluate as broad peaks. To avoid masking of short-chain-homologue peaks the acetonitrile proportion should be decreased, and/or the pH increased.

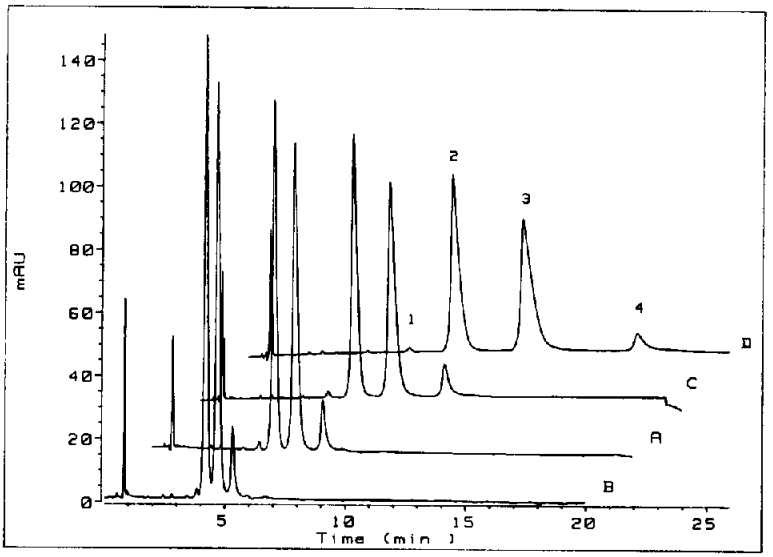

Figure 1
Chromatograms obtained with acetonitrile–TEA (0.1%, v/v) in water (pH 2.5) at different solvent ratios (v/v): (B) 45:55, (A) 40:60, (C) 35:65, (D) 30:70. The peaks shown are: (1) homologue C_{10} (8 μg ml^{-1}), (2) homologue C_{12} (415 μg ml^{-1}), (3) homologue C_{14} (490 μg ml^{-1}), (4) homologue C_{16} (87 μg ml^{-1}).

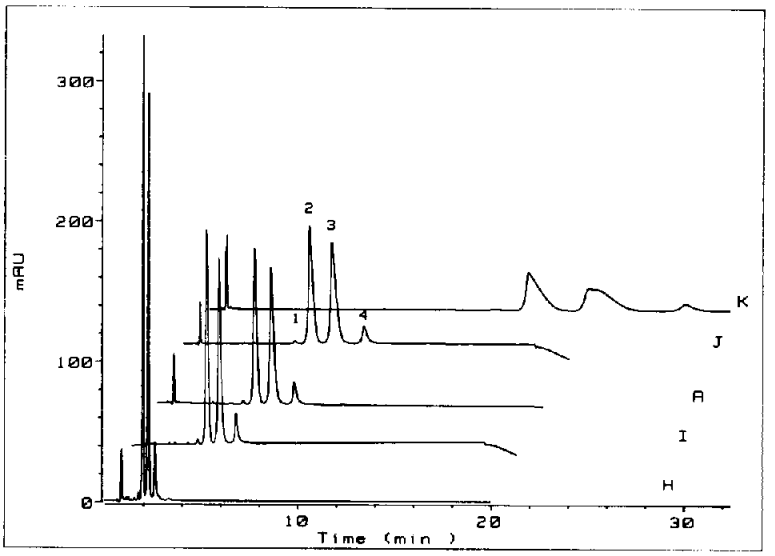

Figure 2
Chromatograms obtained with acetonitrile–TEA in water (pH 2.5) (40:60, v/v) at different TEA concentrations (v/v): (H) 0.5%, (I) 0.2%, (A) 0.1%, (J) 0.05%, (K) 0.00%. Peaks as in Fig. 1.

stationary phase. In consequence, the effect of TEA in the mobile phase is positive, but its concentration is critical.

Effect of aqueous-fraction pH. This effect was tested at pH 2.5–6.0. The lower the pH in this range, the lower the retention and resolution of BAK (Fig. 3). Possibly at an acidic pH, the ammonium group of BAK and the protonated TEA compete for interaction with the stationary phase.

To sum up, the resolution depends on the proportion of organic solvent, the presence or absence of TEA and the pH of the mobile phase; retention is also affected by the concentration of TEA.

The mobile phase (of those tested) that provides acceptable resolution of BAK homo-

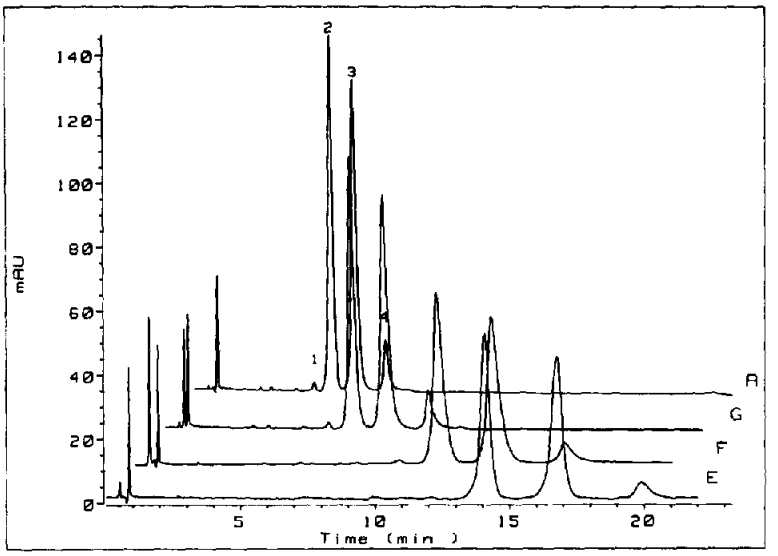

Figure 3
Chromatograms obtained with acetonitrile–TEA (0.1%, v/v) in water (40:60, v/v) at different pH values: (E) pH 6.0, (F) pH 5.0, (G) pH 3.5, (A) pH 2.5. Peaks as in Fig. 1.

Table 3
Selectivity data for the proposed chromatographic conditions

Homologue	K'	W	$T_{0.05}$	Rs
$C_{10}H_{21}$	7.57	0.19	0.79	2.08
$C_{12}H_{25}$	8.60	0.32	1.27	2.42
$C_{14}H_{29}$	10.26	0.39	1.28	3.51
$C_{16}H_{33}$	12.63	0.31	1.45	

K' = capacity factor; W = peakwidth at the baseline (in min); $T_{0.05}$ = tailing factor USP XXII; Rs = resolution.

logues in a short elution time is: acetonitrile–TEA (0.1%, v/v) in water (pH 2.5; 40:60, v/v). Its separation efficiency is summarized in Table 3.

Suitability of the method for the determination of BAK in eye drops

Solutions of common excipients and representative active ingredients were tested with the mobile phase (see Tables 1 and 2).

Under these chromatographic conditions, acidic and non-ionic compounds suffer interaction only slightly with the stationary phase and are eluted almost immediately; this is an advantage in the resolution of BAK.

The amines tested were affected in the same way as was BAK by the presence of TEA in the mobile phase. Under the proposed chromatographic conditions all physiologically active amines tested (see Table 2) were eluted without interference as symmetric and narrow peaks at shorter retention times than that of BAK (practically unretained). When the proportion of acetonitrile was reduced and/or the pH was increased, all peaks were delayed and the simultaneous analysis of this kind of compound and BAK could be carried out.

Some active ingredients (cromolyn sodium and fluorescein sodium) were eluted rapidly but as broad peaks, thus masking the peaks of the short chain-homologues. In these cases the method can be applied by slowing the elution of BAK by increasing the pH or by reducing the proportion of acetonitrile.

Linearity and sensitivity

The linearity of response of BAK (considered as the sum of the homologue areas) was verified at 25–200 ppm (seven different concentrations and $n = 24$). A rectilinear relationship between peak area and concentration was obtained under the proposed chromatographic conditions: peak area = 3.422 [BAK ppm] − 9.720; the correlation coefficient (r) was 0.9995, the intercept did not significantly differ from zero and the relative standard deviation (RSD) for the slope was 0.002.

The sensitivity of response was shown not to depend on the pH of the mobile phase. A slight variation in the slope of the line with the BAK elution time was found to be not significant under the range of conditions tested. The

lower limit of determination was 4 ppm corresponding to 10 times the standard deviation of the ratio signal-to-noise.

Precision

The within-day precision was verified for all standard solutions used in the calibration. RSD values of 0.003–0.057% were obtained over the range of 200–25 ppm of BAK ($n = 3$).

The between-day precision ($n = 3$ days) was determined for the 100 ppm solution; RSD was 0.034%.

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