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Development and validation of a capillary zone electrophoresis method for the determination of benzalkonium chlorides in ophthalmic solutions

Ya-Hui Hou, Chien-Yi Wu, Wang-Hsien Ding*

Department of Chemistry, National Central University, Chung-Li, 32054 Taiwan

Abstract

A systematic investigation of optimal conditions for determining the benzalkonium chlorides in ophthalmic products by capillary zone electrophoresis (CZE) was presents. The most effective separation conditions was 40 m*M* phosphate buffer with 40% acetonitrile at pH 4.0, and the sample hydrodynamic injection of up to 10 s at 2 p.s.i. (1 p.s.i.=6892.86 Pa) (approximately 35.15 nl), and an applied voltage of 15 kV. The reproducibility of the migration time and quantitative analysis can be improved by using internal standard, triethylbenzylammonium chloride, giving the relative standard deviation less than 0.2% for the relative migration times, and 5.0-7.8% for the relative peak areas. A good linearity of CZE analysis was obtained in the range of 1.0–20 μg/ml with r^2 values of above 0.99. The established HPLC with UV–Vis detection was applied to evaluate the CZE method, and compatible results were obtained by using CZE with much shorter analysis time and a small quantity of solvents consumed.

Keywords: Validation; Ophthalmic solutions; Benzalkonium chlorides

1. Introduction

Benzalkonium chlorides (BAKs) are bactericidal antimicrobial agents, which are commonly used in a widely variety of health care and cosmetic preparations. Over 65% of the ophthalmic products currently available on the market use BAKs as the preservatives [1]. They are also used as topical antiseptic and medical equipment disinfectants. The BAKs are a mixture of alkylbenzyldimethyl ammonium chlorides with the general formula $[C_6H_5CH_2N(CH_3)_2R]^+Cl^-$, where R=*n*-$C_8H_{17}$ to *n*-$C_{19}H_{39}$. The *n*-$C_{12}H_{25}$, *n*-$C_{14}H_{29}$, and *n*-$C_{16}H_{33}$ homologs comprise the major portion of the alkyl mixture. Each BAK possess different physical, chemical and microbiological properties. In general, the C_{12} homolog is most effective against yeast and fungi, the C_{14} homolog against gram-positive bacteria, and the C_{16} homolog against gram-negative bacteria [2]. The proportion of these homologs in the mixture determines its effectiveness as a preservative and disinfectant. Therefore, the assay used must be both quantitative and qualitative to be able to identify and distinguish the different BAK homologs and the level of each BAK in the ophthalmic solutions which contain the high concentrations of the major active ingredients.

The determination of BAKs in health care products has been performed by diverse techniques such as two-phase titration [3], gas chromatography [4–7],

*Corresponding author. Tel.: +886-3-422-7151x5905; fax: +886-3-422-7664.

E-mail address: wding@cc.ncu.edu.tw (W.-H. Ding).

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fast atom bombardment mass spectrometry (FAB-MS) [8,9] or more extensively high-performance liquid chromatography (HPLC) [10–16]. Currently, capillary zone electrophoresis (CZE) has become one of the most powerful separation techniques in analyzing large numbers of charged species (see Refs. [17,18] and the references therein). It is preferred in many applications to conventional chromatographic techniques because of its high separation power, small sample volumes, low solvent consumption compared with HPLC, and the possibility of rapid method development (see Refs. [19,20] and the references therein). The CZE has been shown to offer higher resolution separation than does HPLC for ionic surfactants [21–25], and the duration of the analysis is short and uses a small quantity of solvents for separation.

The purpose of this study was to develop and validate a modified CZE method to routinely determine BAK homologs in ophthalmic products, and to compare the results by using the established HPLC method. The influences of CZE separation conditions (i.e. organic modifier content, buffer concentration and pH) were systematically investigated. The use of triethylbenzylammonium chloride (TEA) as an internal standard to improve the peak identification and quantitation results was also demonstrated.

2. Experimental

2.1. Chemicals and reagents

Unless stated otherwise, all high purity chemicals and solvents were purchased from Aldrich (Milwaukee, WI, USA), Tedia (Fairfield, OH, USA) and Merck (Darmstadt, Germany), and were used without further purification. Dodecyl-, tetradecyl-, hexadecyl- and octadecylbenzyldimethyl ammonium chloride (all above 98% purity), decylbenzylammonium chloride (as surrogate) and TEA (as internal standard) were purchased from Aldrich. Sodium dihydrogenphosphate monohydrate (NaH_2PO_4) separation buffer was prepared at stated concentrations between 10 and 50 m*M* in deionized water and were adjusted to stated pH between 3 and 6. The pH of buffers was measured by a Mettler-Toledo MP220 pH meter (Schwerzenbach, Switzerland).

Stock standard mixture containing 1000 μg/ml of each BAK compound in methanol was prepared. Working standard solutions were obtained by diluting the stock standard solution with methanolic solution (70%, v/v) to appropriate concentrations. Separation buffer was prepared with 40% acetonitrile (between 10 and 60%, 40% being optimal, see Section 3) in 40 m*M* NaH_2PO_4 (pH 4.0). Deionized water was further purified with a Minipore water purification device (Millipore, Bedford, MA, USA). Four ophthalmic solution, samples were purchased from local drug stores with prescription and then were diluted with methanolic solution (70%, v/v) directly. Samples 1, 3 and 4 contained 0.1% fluorometholone as a major active ingredient, and sample 2 contained 1.0% tropicamide as a major active ingredient. To prevent capillary blockage, all solutions and samples were filtered through a 0.45-μm membrane filter (Gelman Scientific, Ann Arbor, MI, USA) prior to use.

2.2. CE analysis

All CE experiments were performed on a P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA) equipped with a UV–Vis detector. Separations were carried out in an untreated fused-silica capillary (J&W Scientific, Folsom, CA, USA) of 50 μm I.D. and an effective length of 50 cm (total length 60 cm). The UV detector was operated at 200 nm. All electrophoresis runs were performed at temperature 20 °C. The on-column detection window was made by burning a small section (≈3 mm) of the external polymide coating and scraping off the burned residue with methanol.

Before use, the capillary was conditioned with 1 *M* NaOH for 10 min at 25 °C, followed by 10 min with 0.1 *M* NaOH, 10 min deionized water, and followed by 10 min running buffer. Between runs, the capillary was washed with 0.1 *M* NaOH for 4 min, then 4 min deionized water, and 4 min methanol, and followed by deionized water for 4 min before run. For separation, all samples were hydrodynamically injected into the capillary in 10 s at 2 p.s.i. (1 p.s.i. = 6892.86 Pa), a volume of approxi-

mately 35.15 nl, and an applied voltage +15 kV at the injection end of the capillary.

2.3. HPLC analysis

The procedure used for HPLC analysis has been reported previously [25,26], and was used with minor modifications. Analyses were performed on a HP-1100 high-performance liquid chromatograph system connected to an UV–Vis detector operating at 200 nm. A Hypersil-CPS column (25×0.46 cm I.D., 0.5 μm packing; ThermoQuest, Runcorn, UK) was used at a flow-rate of 2 ml/min, and the injection volume was 20 μl. Isocratic elution was performed with a mixture of 60% acetonitrile and 0.1 *M* sodium acetate adjusted to pH 5.0 with acetic acid. The quantitation of BAKs was carried out using the external standard method to construct four-level calibration curve (or average calibration factor, CF = peak area/amount) covering the range 5–50 μg/ml. The precision of the curve, as indicated by the relative standard deviation (RSD) of calibration factors, was 4.2, 3.5, 5.1 and 4.4% for corresponding C_{12}, C_{14}, C_{16} and C_{18} BAKs. The calibration curves were linear with coefficients of determination $r^2 > 0.99$.

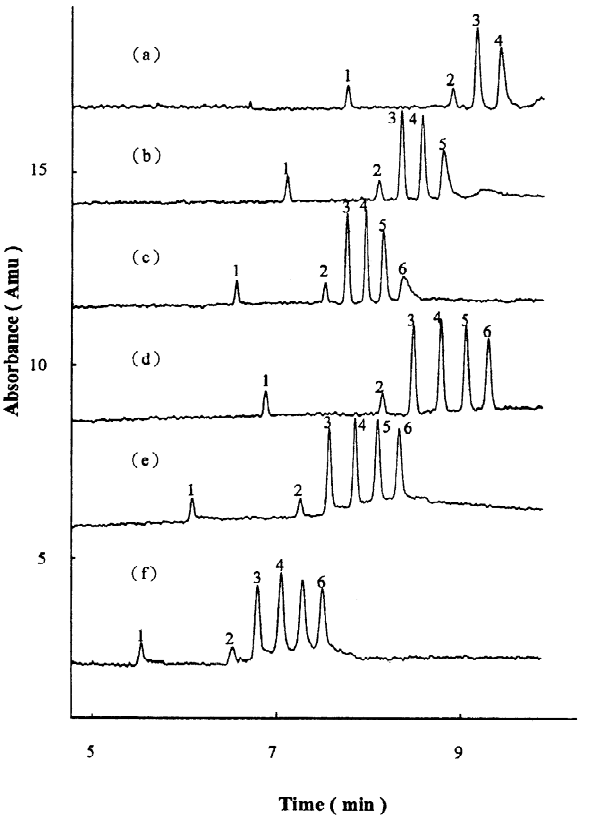

Fig. 1. Separation of BAKs, surrogate and internal standard using acetonitrile in varying proportions (v/v): (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, and (f) 60%. Standard mixture containing 1.0 μg/ml TEA (1, as internal standard); 1.0 μg/ml C_{10} BAK (2, as surrogate); and 5.0 μg/ml each of (3) C_{12}, (4) C_{14}, (5) C_{16}, and (6) C_{18} BAK in deionized water; separation buffer 40 m*M* NaH_2PO_4 (pH 4.0); voltage 15 kV; temperature 20 °C; detection wavelength 200 nm; hydrodynamic injection at 2 p.s.i. for 10 s.

3. Results and discussions

3.1. Evaluation of separation conditions

3.1.1. Acetonitrile content

The formation of micelles and adsorption onto the capillary surface are the critical factors for successful separation of BAKs [21,27]. A simple and effective mean to disrupt micelle formation and to prevent unintended adsorption onto capillary wall is to add an organic modifier into the buffer system. Organic solvents such as methanol [27], tetrahydrofuran [22,23] and acetonitrile [21,28] have been applied as organic modifiers in the CZE buffer system for CZE separation of the BAKs. Among them, acetonitrile (ACN) has been reported as the most effective one [21]. Fig. 1 shows the results of adding different amounts of ACN to the buffer for separation of BAKs, the surrogate and the internal standard (I.S.). With the addition of 10–20% ACN, only I.S. and C_{10}, C_{12} and C_{14} BAK homologs were detected; a weak and diffuse signals of C_{16} and C_{18} BAKs were barely distinguishable from the baseline due to the formation of micelles. As the proportion of ACN was increased, a better separation was achieved. Perfect baseline separation among BAKs was obtained in 12 min when 40% (v/v) ACN was reached (Fig. 1d), which is suitable for obtaining accurate quantitation and calibrations. The resolution was accomplished better than the data reported in previous work [21]. When more ACN was added, all analytes were moved faster but the resolution de-

clined due to the increasing the electroosmotic flow (EOF).

3.1.2. Buffer concentration and pH

The influence of the buffer concentration on the migration time and separation of BAKs was investigated in the range between 10 and 50 m*M* of phosphate buffer at pH 4.0 with 40% of ACN. A better peak resolution was obtained as the proportion of phosphate concentration was increased (Fig. 2). Perfect baseline resolution and better peak shapes were obtained at 30–40 m*M* concentration (Fig. 2c,d). The resolution and peak shape for the BAKs were not significantly different at concentration at 40–50 m*M*.

Buffer pH also plays an important role in CZE separation because it affects both the charge of the analyte and the strength of EOF. The mobility of cationic compounds increased significantly with increased pH from 3 to 6 (Fig. 3). However, in order to decrease or minimize adsorption of cationic surfactants onto the capillary wall due to coulombic interactions, used the lower pH of the buffer was suggested. Here, the buffer pH of 4.0 was selected to decrease the effective negative charge at the wall.

In this study, the most effective separation was achieved by 40 m*M* phosphate buffer with 40% acetonitrile at pH 4.0, and the sample hydrodynamic injection of up to 10 s at 2 p.s.i. (approximately 35.15 nl), and operating at 15 kV and 20 °C.

3.2. Validation of the CZE procedure

To validate the performance of the CZE separation, the reproducibility (in terms of relative standard deviation, RSD) and linearity with standard

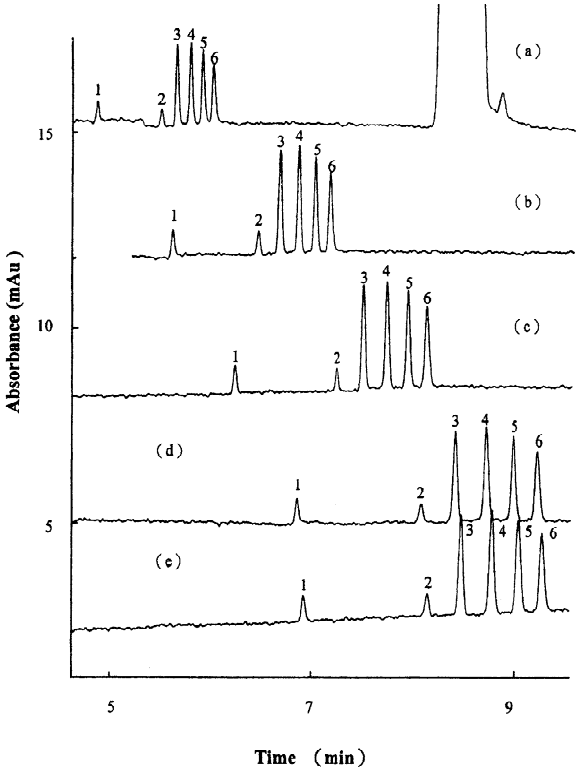

Fig. 2. Comparison of separation and peak shape of BAKs among different buffer concentrations: (a) 10, (b) 20, (c) 30, (d) 40, and (e) 50 m*M*. Peak numbering and experimental conditions as given in Fig. 1.

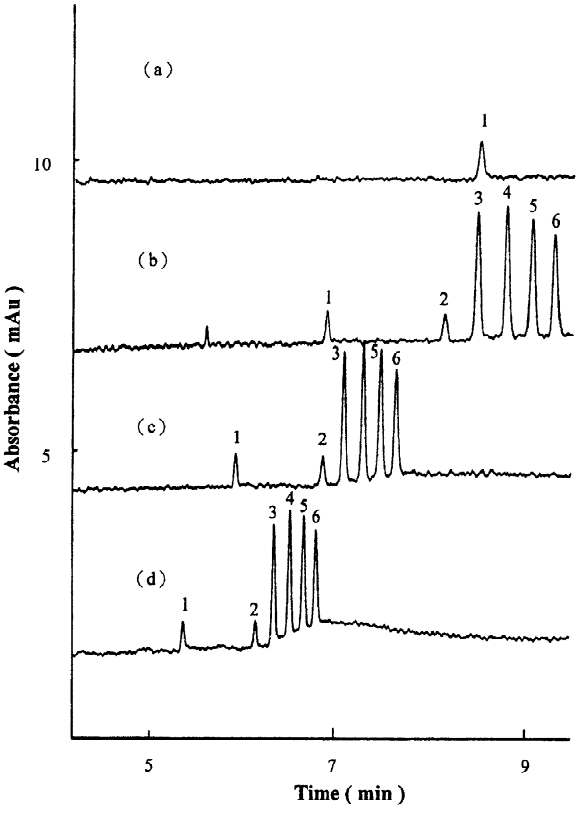

Fig. 3. Effect of pH on the separation of BAKs: (a) pH 3, (b) pH 4, (c) pH 5 and (d) pH 6. Peak numbering and experimental conditions as given in Fig. 1.

Table 1
Reproducibility, linearity of response and response factors using CZE

	BAKs				
	C_{10}(surr.)	C_{12}	C_{14}	C_{16}	C_{18}
(1) Reproducibility ($n=5$, using 1.0 μg/ml each of analytes)					
Migration time (RSD, %)	0.4	0.4	0.4	0.5	0.5
Peak area (RSD, %)	28	25	24	24	23
Relative migration time (RSD, %) (I.S. TEA, 1.0 μg/ml)	0.1	0.2	0.2	0.2	0.2
Relative peak area (RSD, %)	6.3	6.3	5.9	5.0	7.8
(2) Linearity of response					
Correlation coefficient (r^2)	0.993	0.991	0.996	0.995	0.997
Response factor (RSD, %) (I.S. TEA, 1.0 μg/ml)	7.0	9.5	5.2	3.3	6.0
Relative migration time (RSD, %) (concentrations range 1.0–20 μg/ml, 5-level)	0.3	0.4	0.5	0.6	0.6

solution mixtures under the optimum conditions described above was studied. Table 1 summarizes the RSD in migration times, peak areas, relative migration times and relative peak areas, as well as linearity of response. The reproducibility of the technique was tested using five replicate injections of BAK standard mixture (1.0 μg/ml each). The RSD of the migration times and peak areas were around 0.4–0.5 and 23–28%, respectively; however, they were improved significantly when internal standard was used, especially for the RSD of peak areas (5.0–7.8%). Calibration for BAKs was performed in the concentration range between 1.0 and 20 μg/ml (in five-level). Using TEA as an internal standard, the response factors were calculated by the peak areas of BAKs relative to fixed concentration of TEA. The precision of the curve, as indicated by the RSD of response factors ranged from 3.3 to 9.5%. The calibration curves were linear with coefficients of determination $r^2>0.99$. The RSD of the relative migration time was around 0.4–0.6% when various concentrations of BAKs were injected. These results

Table 2
Results and comparison of BAKs determination in ophthalmic solutions using CZE and HPLC

Sample	BAKs		$t_{calculated}$ [a]
	C_{12}	C_{14}	
1 (by CZE)	60	33	0.81
1 (by HPLC)	89	30	
2 (by CZE)	50	33	0.70
2 (by HPLC)	73	29	
3 (by CZE)	45	25	0.74
3 (by HPLC)	65	22	
4 (by CZE)	53	33	0.62
4 (by HPLC)	70	29	
Estimated of limit of quantitation (mg/l)			
CZE	0.5	0.5	
HPLC	2.0	2.0	

$$t_{calculated} = \frac{\bar{d}}{s_d}\sqrt{n} \qquad s_d = \sqrt{\frac{\sum (d_i - \bar{d})^2}{n-1}} d_i$$

is the individual differences between results for each sample; d is the average difference between methods A and B; n is the number of pairs of data (two in this study).

[a] From Ref. [29], Eqs. (4–10) and (4–11).

demonstrate that the CZE analysis for BAKs provides high reproducibility and excellent linearity.

3.3. Applications

The versatility of the validated CZE method is demonstrated in Table 2, which lists the concentrations of BAKs detected in ophthalmic products. Fig. 4 shows the typical electropherograms of CZE obtained for BAKs standards and real samples of ophthalmic solutions. The presence of the C_{12} and C_{14} homologs were detected in all ophthalmic products, C_{12} homolog being the major product in the samples, probably due to its greater disinfecting ability. The RSD of the relative migration time was around 0.5%. The peak identification and quantitation were performed by relative migration times and response factors, respectively, using TEA as an internal standard. The BAK contents of the samples were calculated as the sum of these two compounds, which were in agreement with the manufacturers specification (0.01%), except sample 3. An intense unidentified peak (probably some unidentified inactive ingredients or impurities) was observed in sample 3 (Fig. 4d), which may interfere the calculation of the content of BAKs during the production process. Table 2 also compares the quantitative results obtained from the CZE and the HPLC method. Using the Student's *t*-test procedures to compare individual differences [29], the two methods for BAKs determination were evaluated. In this test, the CZE and HPLC methods were used to make single measurements on several samples, such that no measurement was duplicated. The *t*-test was to determine whether the two methods yielded the same results ''within experimental error'', or was one systematically different from the other in certain confidence level. The calculated-*t* values (in Table 2) were less than the table list *t*-value (12.7) [29] at 95% confidence with one degree of freedom. Therefore, these two methods are not significantly different at the 95% confidence level. However, CZE technique consumed less than 200 μl of solvent for each analysis, whereas HPLC consumed around 50 ml. The sensitivity of the CZE is better compared to established HPLC methods.

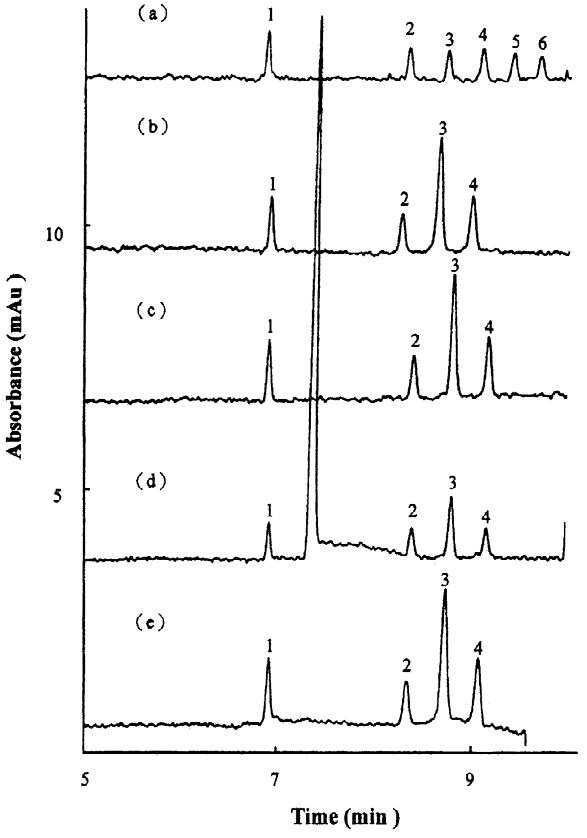

Fig. 4. Electropherograms for the separation of BAKs in ophthalmic products: (a) standard mixture (1.0 μg/ml each of BAKs, surrogate and I.S.), (b) sample 1 (from Alcon-Couvreur), (c) sample 2 (from Alcon-Couvreur), (d) sample 3 (from Ciba Vision), and (e) sample 4 (from Alcon-Couvreur). Peak numbering and CZE operating conditions as in Fig. 1.

In conclusion, the analytical procedure developed herein demonstrates that CZE is a reliable and sensitive method, and offer a convenient analytical technique for determining BAKs in ophthalmic solutions. In separating BAKs by CZE, acetonitrile content, buffer concentration and pH are the three important separation parameters which most affect the migration time and the resolution of BAKs. As expected, CZE analysis leads to better peak shapes, higher efficiency and sensitivity, and consumes significantly less solvent than is required in HPLC analysis. The reproducibility of the migration time and the quantitative results of CZE can be improved by internal standard. These results indicate that CZE method has the potential to become a more efficient and more useful method for BAKs analysis than established HPLC method, and can be applied to the

quantitative analysis as well as qualitative analysis in pharmaceuticals.

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