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Simultaneous determination of ingredients in an ointment by hydrophobic interaction electrokinetic chromatography

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Abstract

Hydrophobic interaction electrokinetic chromatography was used to simultaneously determine seven active ingredients (diphenhydramine hydrochloride, dibucaine hydrochloride, chlorhexidine hydrochloride, phenylephrine hydrochloride, hydrocortisone acetate, allantoin and tocopherol acetate) in an ointment. Not only hydrophobic but also ionic compounds were successfully separated by use of a separation solution composed of acetonitrile–water (80:20, v/v), tetradecylammonium salt and ammonium chloride. The migration behavior of the hydrophobic compound depended on tetradecylammonium concentration, while that of the ionic compounds depended on ammonium chloride concentration. An addition of triethylamine to the separation solution markedly improved the reproducibility of the peak areas of cations with a relative standard deviation (RSD) of less than 1.7% ($n=6$). The established method was validated and confirmed to be applicable to the determination of the active ingredients in a commercial ointment. Sample preparation was performed by liquid–liquid extraction and no interference from the formulation excipients was observed. Good linearities were obtained, with correlation coefficients above 0.999. Recoveries and precisions ranged from 98.0 to 100.8%, and from 0.4 to 2.9% RSD, respectively. These results suggest that hydrophobic interaction electrokinetic chromatography can be used for the determination of ionic compounds as well as hydrophobic compounds in ointment.

Keywords: Ointment; Diphenhydramine; Dibucaine; Chlorhexidine; Phenylephrine; Hydrocortisone; Allantoin; Tocopherol acetate

1. Introduction

High-performance liquid chromatography (HPLC) has been widely used for the determination of several active ingredients in ointments [1–4], while few electrophoretic studies have been performed. A major problem in analysis of such ingredients is their hydrophobic properties. Hydrophobic compounds may precipitate during electrophoresis due to very low solubility in aqueous buffers, necessitating the use of capillary reconditioning procedures. Previously, such hydrophobic compounds have been separated by micellar electrokinetic chromatography (MEKC) [5,6], cyclodextrin-modified MEKC (CD-MEKC) [7] and microemulsion electrokinetic chromatography (MEEKC) [8–10]. In these systems, several organic solvents such as methanol, acetonitrile and 2-propanol have been added to the separation solution, in order to reduce precipitation as

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well as strong interaction of analytes to the pseudostationary phase. However, the amount of solvents that can be added is limited, since at levels exceeding ca. 20% (v/v), micelles and microemulsions are generally not stable. Moreover, an addition of an organic solvent can cause relatively long separation times. The addition of methanol or 2-propanol greatly extends the elution range, probably due to high viscosity. To overcome this problem, MEEKC was performed in suppressed electroosmotic flow (EOF) condition by utilizing an acidic buffer [11]. In this system, the fat-soluble vitamin A palmitate, vitamin E acetate (tocopherol acetate) and vitamin D_3 were separated within 11 min in order of decreasing hydrophobicity. Non-aqueous capillary electrophoresis (NACE) can be used as an alternative technique for the separation of hydrophobic compounds [12,13]. It is easy to manipulate the separation selectivity of the NACE system by changing the organic solvent, and also the additive, which is soluble only in the organic solvent. Miller et al. [14] demonstrated the effect of charge-transfer complexation on the separation of polycyclic aromatic hydrocarbons (PAHs) in a non-aqueous acetonitrile containing planar organic cations (tropylium and 2,4,6-triphenylpyrylium ions) as additives. Tjørnelund and Hansen separated water-insoluble vitamin K_1 as well as alkylparaben preservatives by NACE systems using propylene carbonate with tetradecylammonium ions (TDA^+) [15]. Several authors have also reported a separation system using tetraalkylammonium ions (TAA^+) or cetyltrimethylammonium bromide as additive in mixed acetonitrile–water medium [16–19]; this system is termed hydrophobic interaction electrokinetic chromatography (HIEKC) [20]. Walbroehl and Jorgenson [16] demonstrated the separation of PAHs and mesityl oxide through proposed solvophobic interaction between solutes and TAA^+. The mobility of the solutes increased with increasing hydrophobicity, and the addition of water to the separation solution increased the degree of interaction. Recently, Pedersen-Bjergaad and coworkers [18,19] reported the separation of fat-soluble vitamins by an HIEKC system with TDA^+ as a pseudostationary phase. Highly hydrophobic nonionic compounds were analyzed with short separation times without any precipitation. They also proposed the possibility of simultaneous analysis of fat- and water-soluble vitamins by the same separation system [19].

In this study, we succeeded in simultaneous determination of seven active ingredients, namely diphenhydramine hydrochloride, dibucaine hydrochloride, chlorhexidine hydrochloride, phenylephrine hydrochloride, hydrocortisone acetate, allantoin and tocopherol acetate (see Fig. 1 and Table 1) in an ointment by use of the HIEKC system. This method was found to be applicable not only to hydrophobic but also to cationic compounds. The effect of TDA^+ and ammonium chloride concentrations on separation was investigated, and possible mechanisms of separation are discussed. In addition to separation, attention was also focused on improvement of the reproducibility of the system. Validation of this method for determination of ingredients in a commercial preparation is also described.

2. Experimental

2.1. Equipment

Capillary electrophoresis (CE) was performed on a $HP^{3D}CE$ instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detection system operating at 230 nm. The capillary compartment temperature was maintained at 20°C. Hydrodynamic injection (3.45 kPa×1 s) at the anodic end of the capillary was used to introduce samples. Fused-silica capillaries (50 μm I.D.; 375 μm O.D.; 40 cm in length to the detector and total length of 48.5 cm) were obtained from Agilent Technologies. A bubble cell capillary arrangement was to increase sensitivity. Prior to each day of use, the capillary was conditioned by rinsing with ethanol (100 kPa×15 min), 0.1 *M* NaOH (100 kPa×15 min), deionized water (100 kPa×15 min), and finally the separation solution (100 kPa×15 min). The capillary was rinsed between runs with the separation solution (100 kPa×3 min). All data were collected and analyzed using HP ChemStation software (Agilent Technologies).

Apparent pH values (pH* [21]) of the separation solution were measured with a pH Meter F-22 and a glass electrode (Horiba, Kyoto, Japan).

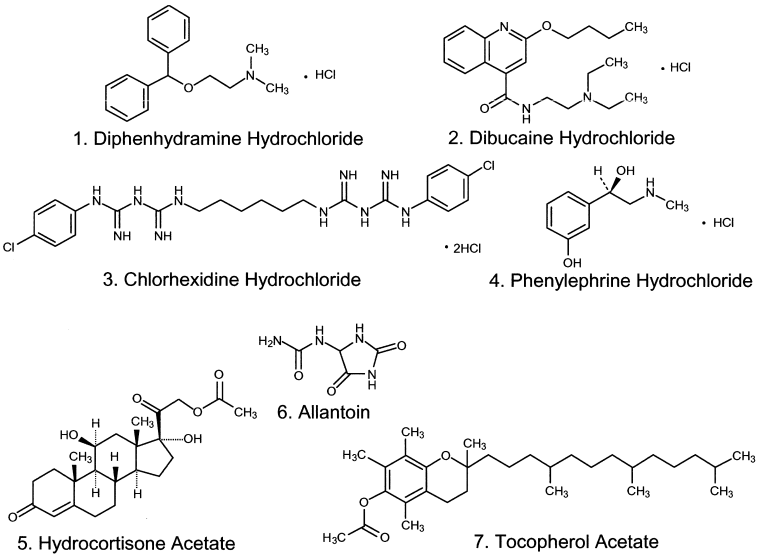

Fig. 1. Active ingredients in an ointment.

2.2. Chemicals

The chemical structures of the active ingredients are shown in Fig. 1. Diphenhydramine hydrochloride was purchased from Kongo Chemincal (Toyama, Japan), dibucaine hydrochloride from Fuji Chemical Ind. (Toyama, Japan), chlorhexidine hydrochloride from Sumitomo Pharmaceuticals (Osaka, Japan), hydrocortisone acetate from the Society of Japanese Pharmacopeia (Tokyo, Japan), allantoin from Kawaken Fine Chemicals (Tokyo, Japan), tocopherol acetate (vitamin E acetate) from the National Institute of Health Sciences (Tokyo, Japan), and butyl parahydroxybenzoate as an internal standard (I.S.) from Welfide (Osaka, Japan). Phenylephrine hydrochloride was obtained from our laboratory (Taisho pharmaceutical, Tokyo, Japan).

Tetradecylammonium bromide was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Triethylamine, acetonitrile, methanol, tetrahydrofuran, formic acid, sodium dodecyl sulphate, octane, 1-butanol, 2-propanol, sodium tetraborate and thiourea were obtained from Wako (Osaka, Japan). Ammonium chloride, acetic acid, ethanol and hexane were obtained from Kokusan Chemical (Tokyo, Japan). All the chemicals were of analytical grade.

Table 1
Content of each active ingredient in an ointment (%)

Active ingredient	Content
Diphenhydramine hydrochloride	0.5
Dibucaine hydrochloride	0.3
Chlorhexidine hydrochloride	0.2
Phenylephrine hydrochloride	0.15
Hydrocortisone acetate	0.3
Allantoin	1
Tocopherol acetate	1

2.3. Preparation of the standard solution for optimization of separation

To prepare the standard ingredient solution for optimization of separation, all compounds except hydrocortisone acetate and tocopherol acetate were initially dissolved in methanol–0.1 *M* formic acid (3:2, v/v). The solution was diluted with methanol, and then about 5 ml of the solution was evaporated by vacuum centrifugation using an Automatic Environmental Speedvac (Savant Instruments, NY, USA). The residue was dissolved in acetonitrile–water (4:1, v/v) at a concentration similar to that present in commercial ointment (40–250 $\mu g\ ml^{-1}$). Hydrocortisone acetate and tocopherol acetate solu-

tions were prepared by dissolving into methanol and ethanol, respectively. All solutions were filtered through a 0.45-μm membrane filter prior to use.

2.4. Procedure for determination of ingredients in a preparation

To determine the ingredients other than tocopherol acetate in a commercial ointment, about 0.5 g of the ointment was weighed accurately into a centrifuge tube. The tube was shaken vigorously after about 15 ml of hexane was added. About 15 ml of methanol–0.1 *M* formic acid (3:2, v/v) was added to the hexane solution and the mixture was shaken for 10 min followed by sonication for 10 min and centrifugation for 5 min at 2000 *g*. The lower layer was transferred to a 50-ml volumetric flask. This extraction process was carried out twice. An internal standard solution was prepared by dissolving 100 mg of butyl parahydroxybenzoate in 100 ml of methanol. Exactly 2 ml of the internal standard solution was added to the solution in the volumetric flask, and the mixture was diluted to the volume with methanol–0.1 *M* formic acid (3:2, v/v). About 5 ml of this solution was evaporated by vacuum centrifugation. The residue was dissolved in 2 ml of the separation solution.

To determine the tocopherol acetate in the commercial ointment, about 0.5 g of the ointment was weighed accurately into a 50-ml centrifuge tube. About 1.5 ml of tetrahydrofuran was added and the tube was shaken vigorously. About 30 ml of ethanol and exactly 2 ml of the internal standard solution were added to the tetrahydrofuran solution in the tube, which was then sonicated for 15 min. The mixture was diluted to a volume of 50 ml with ethanol and centrifuged for 5 min at 2000 *g*. About 5 ml of the supernatant were evaporated by vacuum centrifugation. The residue was dissolved in 2 ml of acetonitrile–tetrahydrofuran (4:1, v/v).

The standard solution for determination was prepared by almost the same procedure as described in Section 2.3, except for the final solvent. The solvent used in standard solution for determination was the same solution as described above. All the test solutions were passed through a 0.45-μm membrane filter.

3. Results and discussion

3.1. Optimization of separation

MEEKC was performed to separate ingredients in an ointment. A microemulsion was prepared by the method reported by Watarai [8]. 2-Propanol was added to the separation solution to reduce precipitation of fat-soluble tocopherol acetate. A typical electropherogram is shown in Fig. 2. The elution range was extended; however, the separation of cationic compounds, such as diphenhydramine hydrochloride, chlorhexidine hydrochloride and dibucaine hydrochloride, were insufficient.

The HIEKC system was applied to the separation of the ingredients in an ointment. Initially, an aqueous solution containing 80% acetonitrile and 80 m*M* TDA^+ was used for separation. Cationic compounds, such as chlorhexidine hydrochloride and phenylephrine hydrochloride, could not be separated (Fig. 3A). Ammonium chloride improved the separation of the cations best among several salts tested. As shown in Fig. 3D, more than 20 m*M* ammonium chloride was required for baseline separation of all compounds. As Porras et al. reported [22], improvement of separation was probably due to an increase in interactions between analytes and the electrolyte anions with an increase in ammonium chloride concentration. Negative two peaks observed behind hydrocortisone acetate and before allantoin were derived from acetonitrile and water, respectively.

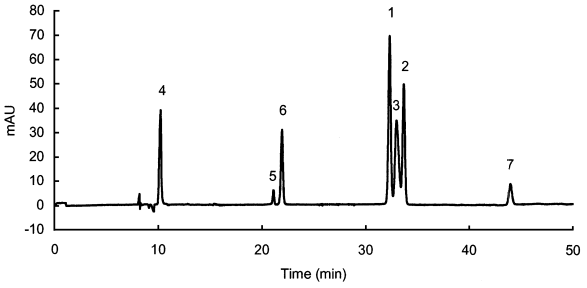

Fig. 2. MEEKC separation of ingredients in an ointment. Separation solution, 0.81% (w/w) octane, 6.61% (w/w) 1-butanol, 10% (w/w) 2-propanol, 3.97% (w/w) sodium dodecyl sulphate and 78.61% (w/w) 10 m*M* sodium tetraborate solution; applied voltage, +15 kV; temperature, 40°C; detection wavelength, 200 nm; capillary, fused-silica (50 μm I.D.×40 cm). Solutes were as noted in Fig. 1.

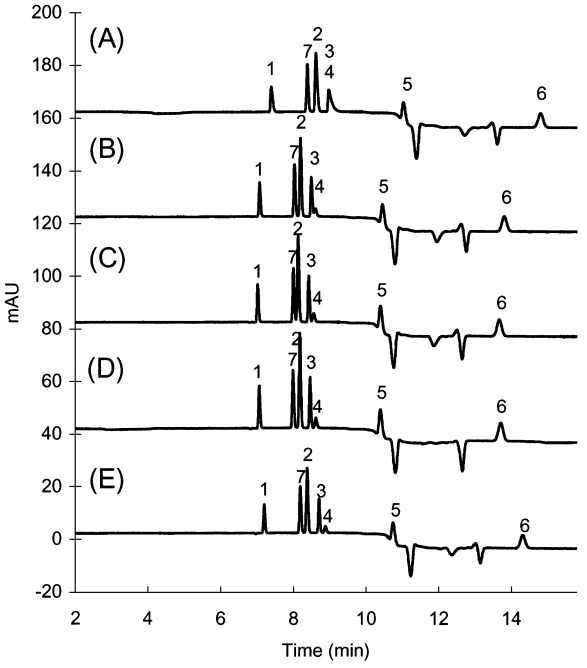

Fig. 3. Effect of ammonium chloride concentration on HIEKC separation of ingredients. Separation solution, (A) no addition, (B) 5 m*M*, (C) 10 m*M*, (D) 20 m*M*, (E) 30 m*M* ammonium chloride and 80 m*M* tetradecylammonium bromide in acetonitrile–water (80:20, v/v); applied voltage, +10 kV; temperature, 20°C; detection wavelength, 230 nm; capillary, fused-silica (50 μm I.D.× 40 cm). Solutes were as noted in Fig. 1.

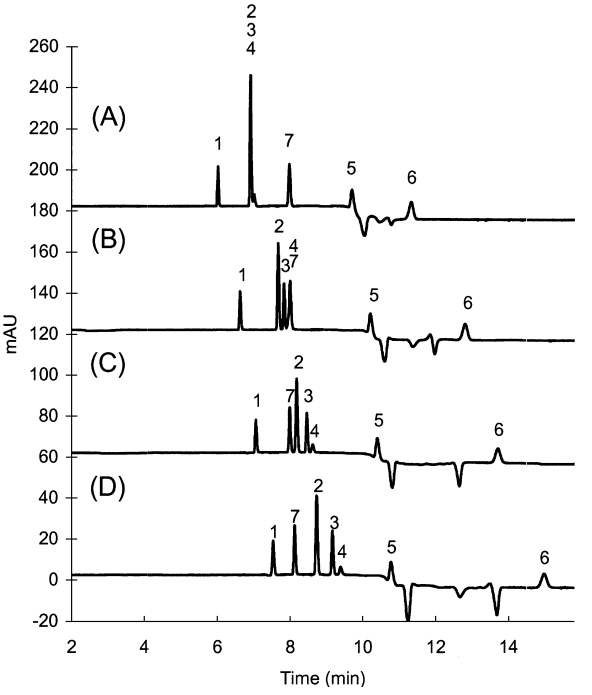

Fig. 4. Effect of TDA^+ concentration on HIEKC separation of ingredients. Separation solution: (A) 40 m*M*, (B) 60 m*M*, (C) 80 m*M*, (D) 100 m*M* tetradecylammonium bromide and 20 m*M* ammonium chloride in acetonitrile–water (80:20, v/v); Solutes were as noted in Fig. 1. Other conditions were as in Fig. 3.

Thiourea, which was used as the EOF marker [15], was detected just behind the latter negative peak. Therefore it is supposed that there is the EOF zone between hydrocortisone acetate and allantoin.

Secondly, the effect of TDA^+ concentration on separation of the ingredients was examined (Fig. 4). When TDA^+ concentration was increased from 40 to 100 m*M*, the EOF decreased as a result of increased viscosity [18], and all ingredients except tocopherol acetate migrated slowly. Enhanced interaction between tocopherol acetate and TDA^+ at higher concentrations of TDA^+ caused the analytes to migrate relatively fast to the EOF. The separation solution composed of 80 m*M* TDA^+ and 20 m*M* ammonium chloride in 80% acetonitrile was therefore selected as optimal for separation. As a result, the HIEKC system modified with ammonium chloride was demonstrated to be effective for separation of all the ingredients including cations in an ointment.

3.2. Reproducibility of the system

To apply the optimal conditions to quantitative analysis, reproducibility of the system was evaluated by six continuous injections of the standard solution. Because of the solubility of each compound, the mixture comprising the standards except for tocopherol acetate was dissolved in acetonitrile–water (4:1, v/v). The cationic compounds including diphenhydramine hydrochloride, dibucaine hydrochloride, chlorhexidine hydrochloride and phenylephrine hydrochloride showed rather poor reproducibility results in the ratio of corrected peak area (relative to I.S.) (Table 2, line 1, and Fig. 5A). The corrected peak area is defined as the ratio of the measured peak area to the migration time of the peak. On the other hand, non-ionic compounds such as hydrocortisone acetate showed very reproducible results. Thus, adsorption of the positively charged analytes onto the capillary wall might be one of the

Table 2
Effect of triethylamine on reproducibility of peak area (RSD% $n=6$)

Separation solution	Sample solvent	Diphenhydramine hydrochloride	Dibucaine hydrochloride	Chlorhexidine hydrochloride	Phenylephrine hydrochloride	Hydrocortisone acetate	Allantoin	Tocopherol acetate
A	C	3.9	3.6	3.7	4.9	1.8	4.0	–
A	A	2.4	2.1	2.2	4.0	1.4	0.9	–
A	D	–	–	–	–	–	–	1.3
B	B	1.1	0.3	1.4	1.7	1.1	0.9	–
B	D	–	–	–	–	–	–	1.0

(A) 80 m*M* tetradecylammonium, 20 m*M* ammonium chloride in acetonitrile–water (80:20, v/v).
(B) 80 m*M* tetradecylammonium, 20 m*M* ammonium chloride, 20 m*M* triethylamine, 40 m*M* acetic acid in acetonitrile–water (80:20, v/v).
(C) Acetonitrile–water (80:20, v/v).
(D) Acetonitrile–tetrahydrofuran (80:20, v/v).

reasons for poor reproducibility. To reduce such adverse effect, a mixture of the standards was dissolved in the separation solution containing a relatively high total concentration of the salts. The use of the separation solution to dissolve a mixture of the standards resulted in slight improvement in peak area reproducibility (Table 2, line 2). Next, triethylamine was added to the separation solution to further improve reproducibility for the cationic compounds. Alkylamines have been used as additives to improve separation efficiency and to reduce the tailing of positively charged analytes in HPLC [23,24] and CE [25–30]. Alkylamines interact primarily with non-derivatized silanol groups by hydrogen bonding, thereby reducing adsorption and/or ion-exchange effects. To investigate the effect of short-chain alkylamine on the reproducibility in the HIEKC system, 20 m*M* triethylamine was added to the separation solution described above. Unfortunately, it was found that the EOF was enhanced, and that diphenhydramine hydrochloride, dibucaine hydrochloride and phenylephrine hydrochloride migrated almost at the same migration times (Fig. 5B). This migration shift was caused by increase in apparent pH of the separation solution (pH* 8.9) due to the addition of triethylamine. In order to retain the baseline separation of all ingredients, 40 m*M* acetic acid was added (Fig. 5C). Final pH* (6.7) was close to initial pH* (5.4). When reproducibility of the system was examined in these conditions, remarkable improvement was observed (Table 2, line 4). These findings suggest that the addition of triethylamine to the separation solution effectively prevents adsorption of positively charged analytes onto the capillary wall in the HIEKC system.

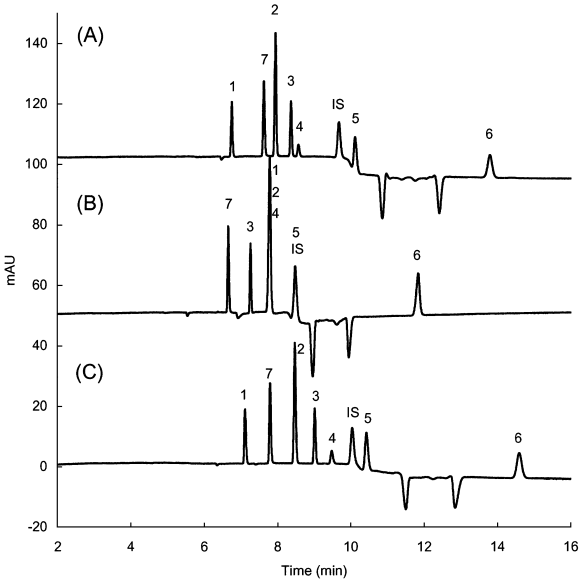

Fig. 5. Effect of triethylamine on HIEKC separation of ingredients. Separation solution: (A) 80 m*M* tetradecylammonium bromide and 20 m*M* ammonium chloride in acetonitrile–water (80:20, v/v); (B) (A) +20 m*M* triethylamine; (C) (B) +40 m*M* acetic acid. Solutes were as noted in Fig. 1. Other conditions were as in Fig. 3.

In addition, tocopherol acetate, which was dissolved in acetonitrile–tetrahydrofuran (4:1, v/v)

because of its solubility, was evaluated for reproducibility of peak area. Reproducible results were obtained, without the adsorption phenomenon (Table 2, lines 3 and 5).

3.3. Determination of the active ingredient

Finally, the separation solution composed of 80 m*M* TDA^+, 20 m*M* ammonium chloride, 20 m*M* triethylamine and 40 m*M* acetic acid in acetonitrile–water (80:20, v/v) was established to give the optimum separation conditions for simultaneous analysis of cationic as well as hydrophobic compounds by HIEKC. The application and validation of this technique were also demonstrated for determination of ingredients in a commercial preparation.

To assess the specificity, each standard, a sample of a commercial ointment and placebo mixtures that were prepared in the absence of each active ingredient in the ointment base were analyzed according to the established method. As described in Section 2.4, the extraction procedures were necessary for analysis of each ingredient in the ointment. A representative electropherogram of each sample solution in Fig. 6 shows the separation between the main peaks and the I.S. In Fig. 6B, many peaks except tocopherol acetate and I.S. were also observed. They were the formulation excipients and other active ingredients extracted insufficiently. No interference from the formulation excipients was observed at the migration times of the ingredients. The applicability of the method as a stability-indicating assay is under further investigation of degradation products.

The detection limit estimated as a peak with a signal-to-noise ratio of 3 and quantitation limit as a peak with a signal-to-noise ratio of 10 are shown in Table 3. Ten replicate injections at the quantitation limit level yielded acceptable relative standard deviation (RSD) values ranging from 4.1 to 8.3% for the ratio of the corrected peak area.

The quantitation linearity of active ingredients in standard solution was examined at five concentration levels in the range from 50 to 150% of the normal concentration. For each ingredient, the relationship between relative corrected peak area and concentration was calculated and is given in Table 3. In all cases, straight regression lines passing through the origin with correlation coefficients (r) above 0.999 were obtained.

Accuracy was assessed over the entire concentration range (80, 100 and 120%) by analyzing placebos spiked with active ingredients at three concentration levels. The solutions were replicated three times each, and the amounts determined were compared to the theoretical amounts. Acceptable results for recovery were obtained for all ingredients studied (Table 3).

Precision was determined by measuring ($n=3$)

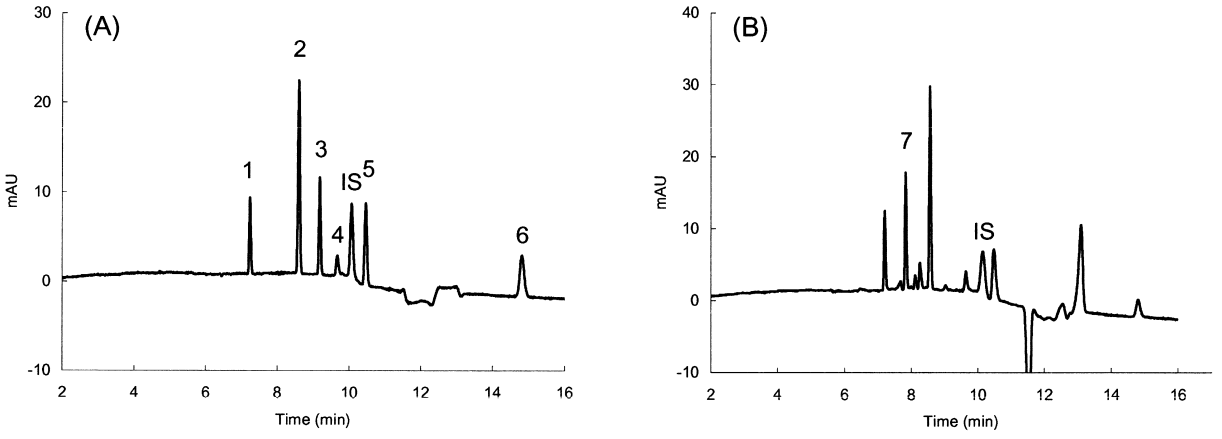

Fig. 6. Typical electropherogram in the assay of a commercial ointment. (A) Methanol–formic acid extract; (B) ethanol extract. Solutes were as noted in Fig. 1. Conditions were as in Fig. 5C.

Table 3
Validation data for determination of active ingredients in a commercial ointment

	Diphenhydramine hydrochloride	Dibucaine hydrochloride	Chlorhexidine hydrochloride	Phenylephrine hydrochloride	Hydrocortisone acetate	Allantoin	Tocopherol acetate
Linearity concentration range (μg ml^{-1})	60–190	40–110	25–75	20–60	40–110	125–375	125–375
r	0.9990	0.9995	0.9991	0.9993	0.9990	0.9993	0.9992
Intercept	−0.0093	0.0437	−0.0120	−0.0070	−0.0046	−0.0146	0.0116
Slope	0.00745	0.02787	0.01917	0.00715	0.01179	0.002403	0.00569
Recovery (n=3, %):							
80%	100.1	99.4	98.9	98.7	98.0	99.9	98.1
100%	100.6	100.1	98.5	98.8	98.8	100.0	99.6
120%	100.8	100.3	99.6	99.4	98.5	99.5	99.0
Precision (n=3, RSD %):							
80%	1.0	0.6	1.7	2.8	1.0	2.3	1.0
100%	0.4	1.6	2.4	1.4	1.2	2.0	0.8
120%	1.3	1.5	2.9	1.8	0.6	2.0	1.6
Detection limit (S/N=3) (μg ml^{-1})	5	1	2	3	3	15	7
Quantitation limit (S/N=10) (μg ml^{-1})	18	4	5	10	8	50	25

each active ingredient in spiked placebos at the three concentration levels. RSD was estimated from the established method. Acceptable results with respect to precision were obtained for all ingredients examined (Table 3).

4. Conclusion

Successful simultaneous determination of not only hydrophobic but also ionic compounds was developed with the single HIKEC system. In order to analyse all the ingredients by HPLC, several conventional methods [2,4] or a gradient elution method may be required. The proposed method has great potential for assay of many types of pharmaceutical ingredients including ionic as well as non-ionic compounds with similar performance level to each HPLC method [1–4]. Thus, the CE method is suitable for simultaneous determination in pharmaceutical quality control. The attractive separation mode, HIEKC, will be a complement to MEKC and MEEKC, and widely extend the area of application of CE.

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