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Separation of fat-soluble vitamins by hydrophobic interaction electrokinetic chromatography with tetradecylammonium ions as pseudostationary phase

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Abstract

Hydrophobic interaction electrokinetic chromatography (HIEKC) was evaluated for the separation of nonionic hydrophobic pharmaceuticals utilizing vitamin A palmitate, vitamin E acetate, and vitamin D_3 as model compounds. In order to effectively dissolve the highly hydrophobic vitamins, the separation medium consisted of acetonitrile–water (80:20, v/v). Tetradecylammonium (TDA^+) at a concentration of 80 m*M* was added to the separation medium as pseudostationary phase, and the three vitamins were separated prior to the electroosmotic flow based on different hydrophobic interactions to the charged TDA^+ molecules. With a 47 cm×50 μm internal diameter uncoated fused-silica capillary, the three vitamins were baseline separated within 6 min utilizing a separation voltage of 10 kV. Migration times were repeatable within 1.3% and varied less than 4.2% between 5 different days. Peak areas were repeatable within 1.7% and varied less than 4.2% between 5 different days. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Hydrophobic interaction electrokinetic chromatography; Pseudostationary phases; Vitamins; Tetradecylammonium ions

1. Introduction

In recent years, capillary electrophoresis (CE) has emerged as an attractive and alternative technique for pharmaceutical quality control, where both ionic and neutral substances may be effectively separated based on their charge, size, and hydrophobicity [1–3]. While substantial research has been devoted to relatively hydrophillic compounds, only little work has been reported on the CE separation of highly hydrophobic nonionic pharmaceuticals. Thus, in order to speed up the implementation of CE within laboratories concerned with pharmaceutical quality control, fast, stable and reliable CE separation systems have also to be developed for the latter type of compounds.

With fat-soluble vitamins as model compounds, CE separation of hydrophobic nonionic pharmaceuticals is highlighted in the present work. Previously, fat-soluble vitamins have been separated by micellar electrokinetic chromatography (MEKC) [4–6] and by microemulsion electrokinetic chromatography (MEEKC) [7]. With the former technique, the fat soluble vitamins were separated based on different partition between the aqueous separation electrolyte

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and micelles as the pseudostationary phase. However, owing to the strong hydrophobic nature of the fat-soluble vitamins, strong interactions with the micelles were evidenced which resulted in very long separation times. This was also the case with MEEKC, where fat-soluble vitamins were separated based on different partition between an aqueous separation electrolyte and microemulsion droplets stabilized by sodium dodecyl sulphate (SDS) or trimethyltetradecylammonium bromide (CTAB).

Hydrophobic interaction electrokinetic chromatography (HIEKC) utilizing acetonitrile–water buffers containing tetraalkylammonium ions as the pseudostationary phase has briefly been proposed as an attractive CE technique for the separation of highly hydrophobic substances [8,9]. With polyaromatic hydrocarbons as model compounds, separations based on different attraction to the charged pseudostationary phase through hydrophobic interactions were achieved. In cases where large amounts of acetonitrile were added to the running buffer, the hydrophobic compounds migrated prior to the electroosmotic flow in decreasing order of hydrophobicity. This reversed order of migration as compared with MEKC and MEEKC seems very attractive for the separation of nonionic hydrophobic pharmaceuticals, and has been evaluated in the present work with vitamin A palmitate, vitamin E acetate, and vitamin D_3 as model substances. In addition to aspects of separation, substantial attention was also focused on system stability and practical considerations.

2. Experimental

2.1. Chemicals

Vitamin A palmitate, vitamin E acetate, vitamin D_3, and tetradecylammonium bromide (99%) were all purchased from Sigma (St. Louis, MO, USA). Acetonitrile, tetrahydrofuran, *n*-hexane (chromatography grades), and sodium hydroxide were from Merck (Darmstadt, Germany), while ethanol (96%) was from Vinmonopolet (Oslo, Norway).

2.2. Preparation of standard solutions and vitamin extracts

A standard solution containing vitamin A palmitate (8 mg/ml), vitamin E acetate (4 mg/ml), and vitamin D_3 (2 mg/ml) was prepared in tetrahydrofuran. The standard solution was protected from exposure to light, and stored at +5°C.

Vitamin extracts of commercial formulations were prepared from both a solid and a liquid product. With the former, a single tablet containing 50 mg vitamin E acetate was pulverized and subsequently extracted for 5 min with 10 ml of tetrahydrofuran on a Whirlimixer. The tetrahydrofuran phase was exposed to 5 min of centrifugation and subsequently injected directly into the CE instrument. For the liquid product, which contained 1 mg/ml of vitamin E acetate, 3 ml vitamin formulation was extracted for 10 min with *n*-hexane on a Whirlimixer. Following 5 min of centrifugation, the *n*-hexane phase was evaporated and subsequently redissolved in 0.4 ml tetrahydrofuran prior to injection.

2.3. Apparatus and separations

The CE-system was a P/ACE 5510 Series from Beckman (Fullerton, CA, USA) equipped with a diode array detector. Separations were performed inside 50 μm I.D. uncoated CElect fused-silica capillaries from Beckman with the detector window placed 7 cm from the capillary outlet. The effective length of the separation capillaries was either 40 or 60 cm. The polyimide coating was removed from both ends of the capillary, in order to prevent dissolution in the highly organic electrolyte systems. The cathode was placed at the outlet of the capillary. Sample introduction was accomplished by hydrodynamic injection with pressure (0.5 p.s.i., 1 s, 1 p.s.i.=6894.76 Pa). The compounds were detected on-column at 238 nm with an aperture of 100×800 μm. Electropherograms were recorded and processed with the capillary electrophoresis software for the P/ACE System 5000 Series (Beckman). The capillary was conditioned prior to use each day by rinsing for 10 min with ethanol, for 20 min with 1 *M* sodium hydroxide, for 25 min with water, and finally for 3 min with running buffer.

3. Results and discussion

3.1. Separation strategy

HIEKC with tetraalkylammonium ions as the pseudostationary phase has previously been used for the separation of polyaromatic hydrocarbons (PAHs) in mixed acetonitrile–water media [8,9]. Due to different hydrophobic interactions of the PAHs with the positively charged tetraalkylammonium ions, the uncharged and highly hydrophobic substances were effectively separated within the CE system. In both publications, attention was primarily focused on separation with electrolyte systems containing up to 50% acetonitrile, and with 10–25 m*M* tetraalkylammonium ions as the pseudostationary phase. Owing to this relatively low content of acetonitrile in the separation buffer, the solubility of the tetraalkylammonium ions was relatively low and some of the pseudostationary phase was bound to the wall of the fused-silica. This caused a reversal of the capillary charge from negative (silanol groups) to positive, and following this the electroosmotic flow (EOF) was reversed. In this type of separation system, where the anode was placed at the outlet of the capillary, the neutral compounds migrated after the EOF in order of increasing hydrophobicity. Besides this upstream mode, a downstream mode was proposed [9] where the amount of acetonitrile was increased. With the latter separation electrolyte, the solubility of the pseudostationary phase increased, and the tetraalkylammonium ions were released from the capillary wall. Thus, in the downstream mode, the capillary carried a negative surface charge, and the direction of the EOF was normalized with the cathode placed at the outlet of the capillary. In this downstream mode, all neutral compounds migrated prior to the EOF in order of decreasing hydrophobicity. For the separation of hydrophobic nonionic pharmaceuticals, this type of downstream system with short migration times for the most fat-soluble compounds was considered to be very interesting, and was consequently studied and evaluated below with three fat-soluble vitamins as model compounds (Fig. 1).

Because speed of analysis was so important in the present work, both acetonitrile and N-methylformamide were considered as organic modifiers for the separation electrolyte. In both cases, the high ratio between the dielectric constant and the viscosity results in relatively high electroosmotic mobilities as compared with other potential solvents like methanol, ethanol, and dimethyl sulfoxide [10]. However, while mixtures of acetonitrile and water were found to provide acceptable solubility of TDA^+ which was selected as pseudostationary phase, the solubility of TDA^+ was very low in N-methylformamide–water media. Thus, acetonitrile–water was utilized in the present work as separation electrolyte. TDA^+ provided the largest hydrophobic area among the tetraalkylammonium bromides commercially available. This was desired in order to effectively stimulate hydrophobic interactions between the hydrophobic pharmaceuticals and the pseudostationary phase and to obtain the required separation.

3.2. Optimization of the separation

For vitamin A palmitate, vitamin E acetate, and vitamin D_3, the separation systems proposed for PAHs in the literature with 50–60% acetonitrile and 10–20 m*M* TDA^+ in the separation electrolyte [8,9] failed because the vitamin A component precipitated within the separation capillary. When the content of acetonitrile was increased to 70%, the solubility increased and a peak for vitamin A palmitate appeared in the electropherogram. In spite of this, 10–20 m*M* TDA^+ was insufficient to effectively accomplish separation of the vitamins. As the TDA^+ level was increased to 50 m*M* however, hydrophobic interactions were stimulated and comigration between the model compounds was avoided. With this separation system, all three vitamins migrated prior to the EOF in order of decreasing hydrophobicity when the cathode was placed at the outlet of the capillary. This finding was in accordance with results reported recently for the separation of some PAHs [9]; at high levels of organic modification, the pseudostationary phase was easily dissolved by the separation electrolyte and binding to the capillary wall was suppressed. In order to further improve the separation system, careful optimization was carried out.

In a first experiment, the degree of organic modi-

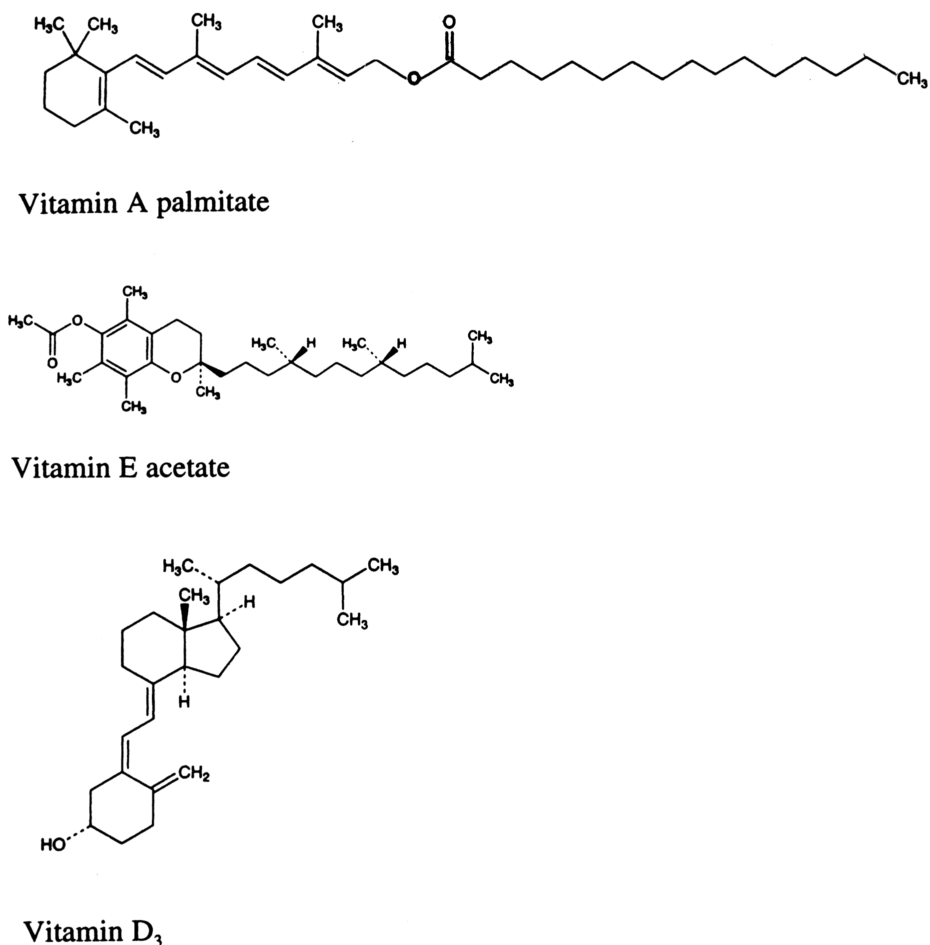

Fig. 1. Structures of vitamin A palmitate, vitamin E acetate and vitamin D_3.

fication was optimized by variation of the acetonitrile content from 70 to 90%. At least 70% acetonitrile was required to effectively dissolve the vitamins within the separation electrolyte, while the solubility of TDA^+ decreased significantly as the amount of acetonitrile approached 100%. As illustrated in Fig. 2, all the three vitamins were effectively separated within the CE system when the amount of acetonitrile was within 70 and 85%. As the amount of organic modifier was increased further, hydrophobic interactions between the vitamins and the pseudo-stationary phase were suppressed. Thus, with 90% acetonitrile, the separation between vitamin A palmitate and vitamin E acetate deteriorated. Based on this experience, it was decided to use a final separation electrolyte with 80% acetonitrile and 20% water.

In a second experiment, the amount of pseudo-stationary phase was varied in order to find the optimal level. At least 50 m*M* of TDA^+ was required to obtain acceptable separation of the three fat-soluble vitamins. As the concentration was further increased from 50 to 80 m*M* (Fig. 3), the resolution between the peaks improved somewhat. As the concentration of TDA^+ was increased above 80 m*M* however, the EOF decreased owing to an increased viscosity while no further effect was observed on the separation. Thus, 80 m*M* was selected as the optimal level of TDA^+ for the vitamin separation.

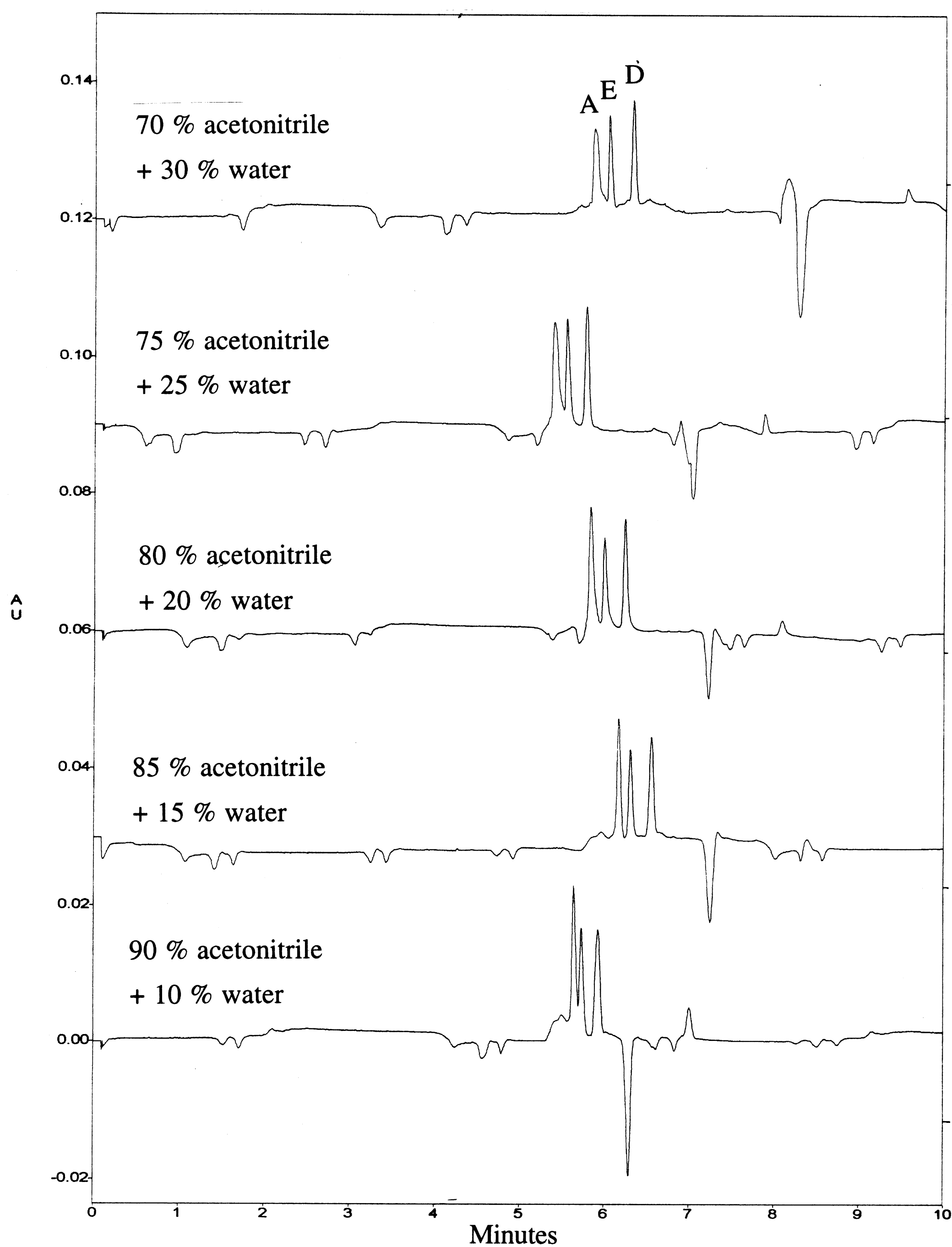

Fig. 2. Separation of vitamin A palmitate (8 mg/ml), vitamin E acetate (4 mg/ml), and vitamin D_3 (2 mg/ml) by HIEKC. Separation electrolyte: 80 m*M* tetradecylammonium bromide in different acetonitrile–water mixtures. Capillary: 47 cm×50 μm I.D; separation voltage: 10 kV; detection: 238 nm.

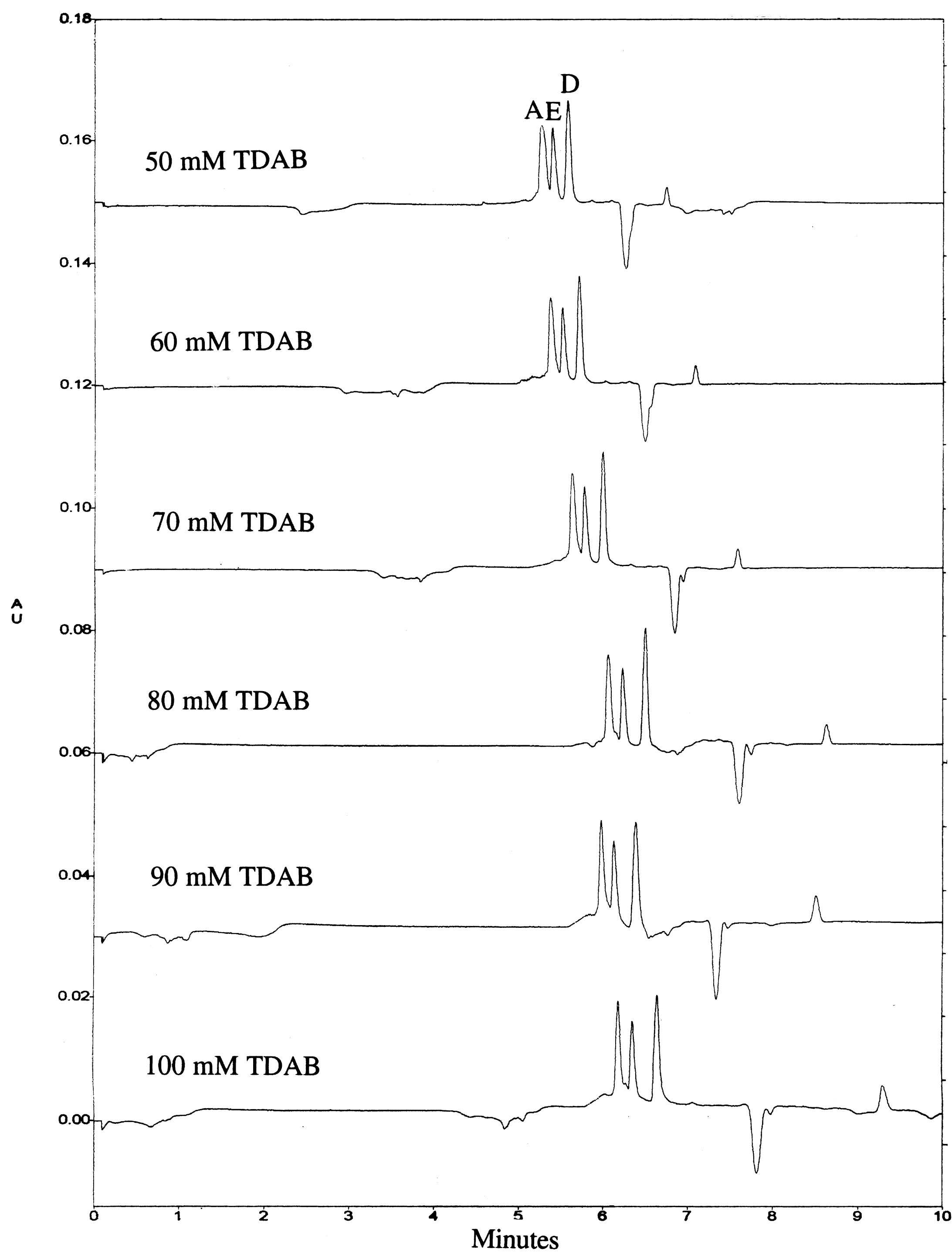

Fig. 3. Separation of vitamin A palmitate (8 mg/ml), vitamin E acetate (4 mg/ml), and vitamin D_3 (2 mg/ml) by HIEKC. Separation electrolyte: acetonitrile–water (80:20, v/v) and different concentrations of tetradecylammonium bromide. Capillary: 47 cm×50 μm I.D.; separation voltage: 10 kV; detection: 238 nm.

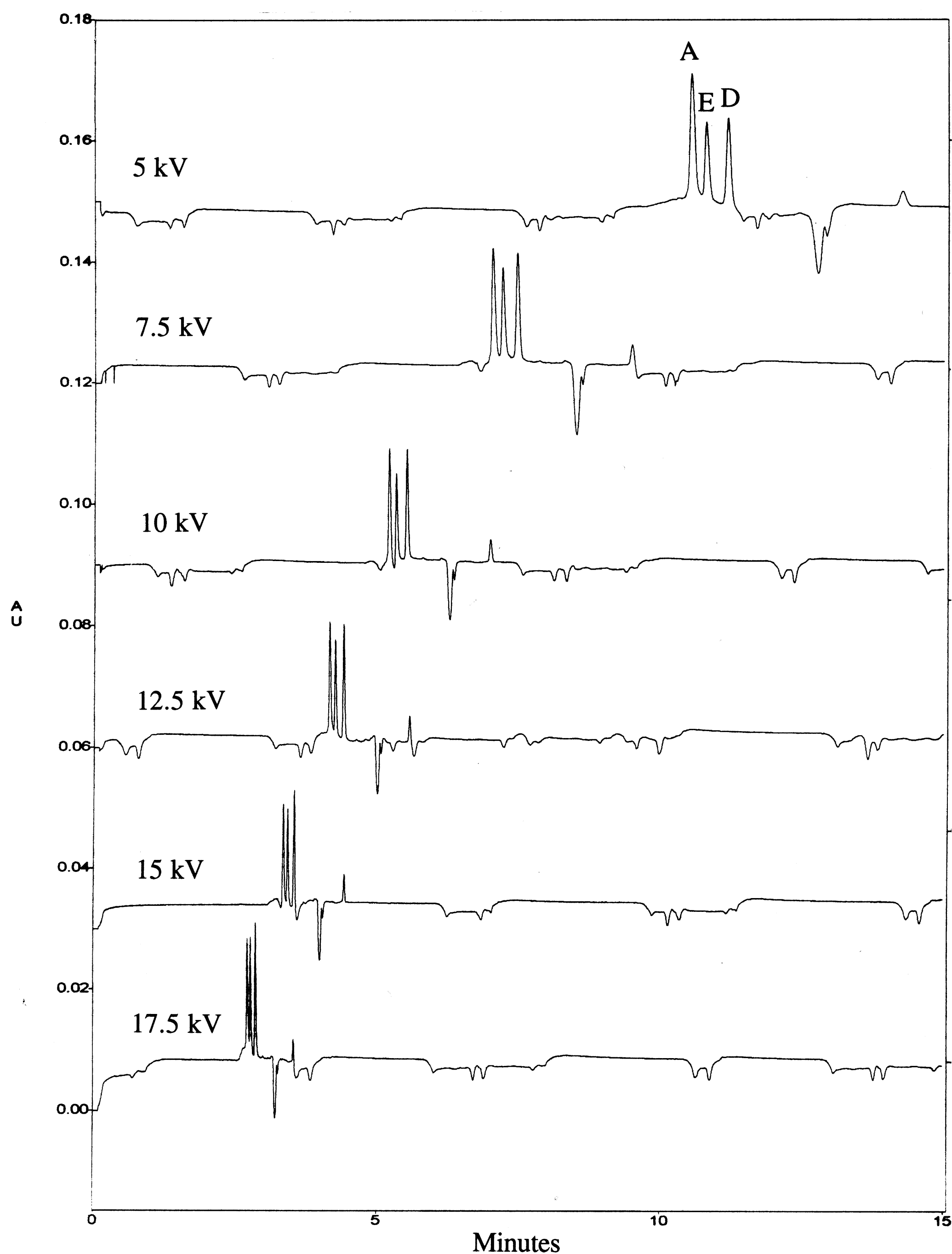

Fig. 4. Separation of vitamin A palmitate (8 mg/ml), vitamin E acetate (4 mg/ml), and vitamin D_3 (2 mg/ml) by HIEKC. Separation electrolyte: acetonitrile–water (80:20, v/v) and 80 m*M* tetradecylammonium bromide. Capillary: 47 cm×50 μm I.D.; different separation voltages; detection: 238 nm.

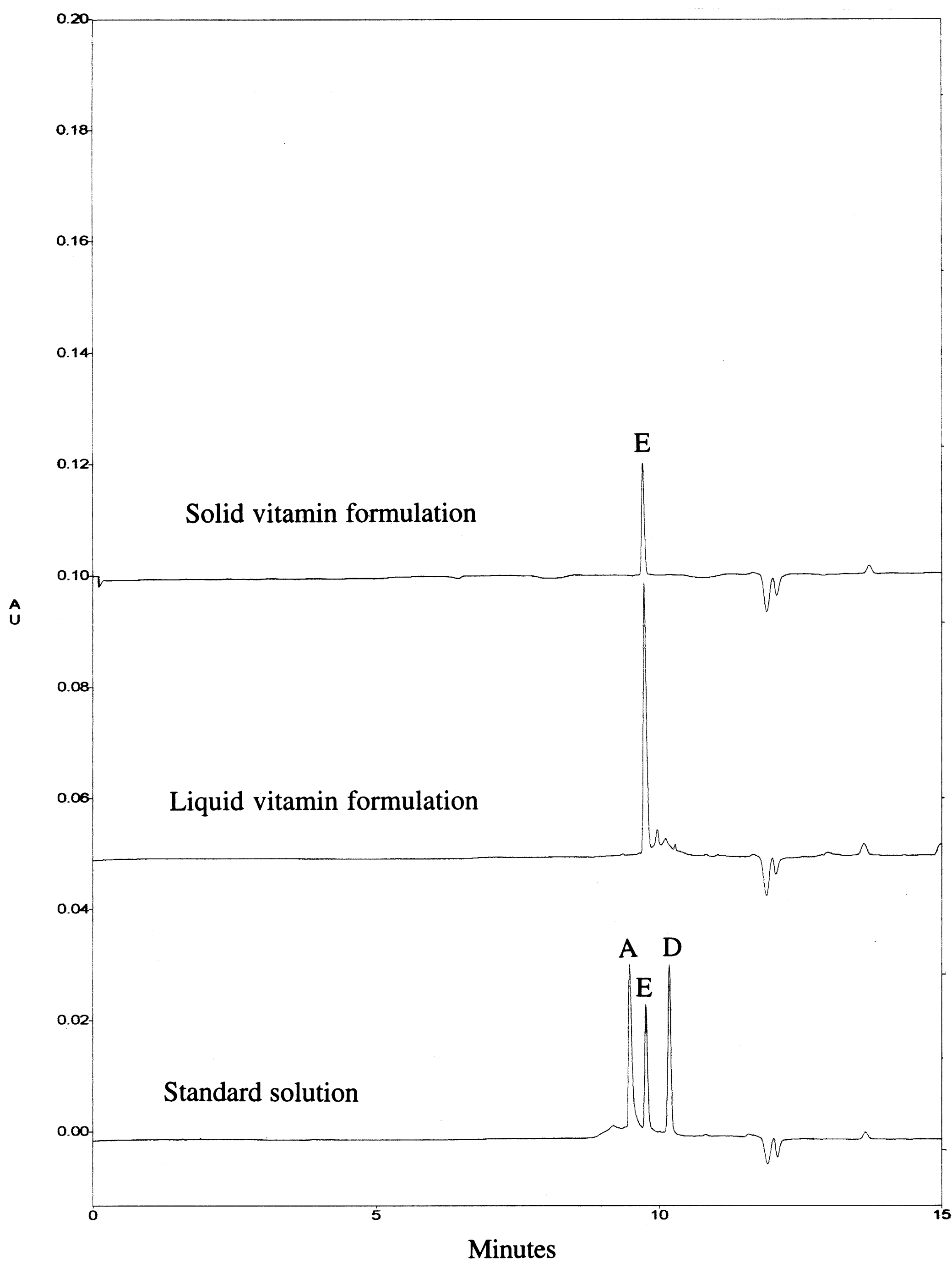

Fig. 5. Analysis of commercial vitamin formulations by HIEKC. Separation electrolyte: acetonitrile–water (80:20, v/v) with 80 m*M* tetradecylammonium bromide. Capillary: 67 cm×50 μm I.D.; separation voltage: 10 kV; detection: 238 nm.

In addition to the buffer composition, the vitamin separation was optimized with respect to separation voltage, which varied from 5 to 17.5 kV. Below 5 kV, the EOF was very low resulting in long separation times, while serious current problems were observed as the voltage exceeded 17.5 kV. As expected, the time of analysis decreased dramatically as the separation voltage increased from 5 to 17.5 kV (Fig. 4). Up to 15 kV, the resolution between the vitamins were almost unaffected as the voltage was increased, while some comigration was observed between vitamin A palmitate and vitamin E acetate at 17.5 kV. Because current problems occasionally occurred even at 15 kV, 10 kV was selected as the operating separation voltage to ensure high system stability.

Operating at 10 kV with a acetonitrile–water (80:20, v/v) separation buffer, and 80 m*M* TDA^+, the vitamins were separated within 6 min utilizing a 40 cm (effective length)×50 μm I.D. column. For practical work with real vitamin products, where baseline separation was of vital importance, the effective length of the capillary was increased to 60 cm (Fig. 5). In this capillary, the vitamins were separated within 9 min at 10 kV, and the system provided between 125 000 and 200 000 theoretical plates for the selected model compounds.

3.3. Practical considerations

Because of the high organic modification of the separation electrolyte, some practical considerations were very important in the present work compared with conventional CE in aqueous media. Firstly, the separation electrolyte was found to dissolve the outside polyimide coating of the fused-silica capillary. Thus, during operation, the separation electrolyte and the capillary were both gradually contaminated which resulted in poor control of the EOF and background problems during UV diode array detection. This problem, however, was effectively eliminated when the polyimide coating was burned off at both ends of the capillary exposed to the separation electrolyte.

During the first experiments, standard solutions of the three fat-soluble vitamins were prepared in acetonitrile modified with 10% tetrahydrofuran (THF). The latter component was utilized in order to increase the solubility of vitamin A palmitate, which was difficult to dissolve in pure acetonitrile. In spite of the THF modification, the most hydrophobic component vitamin A palmitate easily precipitated on the uncoated outside surface of the fused-silica capillary. Thus, following injection when the capillary was repositioned into the separation electrolyte, vitamin A palmitate was gradually dissolved into the separation electrolyte which resulted in poor electrophoretic resolution and serious background problems during UV diode array detection. This problem was effectively solved by preparing the standard solutions in pure THF which gave excellent solubility of the vitamins, and no precipitation was observed at the uncoated end of the capillary.

Since the EOF was important in the control of migration times, the capillary was flushed for 10 min

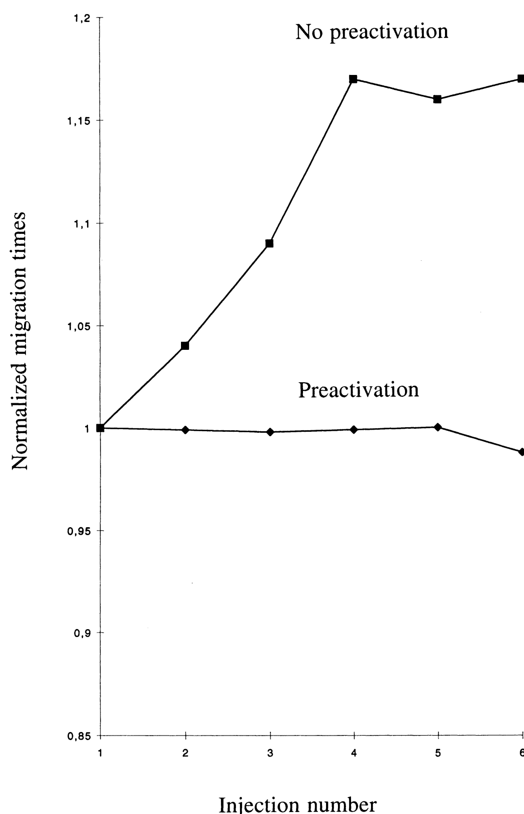

Fig. 6. Effect of capillary preactivation between each run on migration time stability (4 mg/ml vitamin E acetate). Separation electrolyte: acetonitrile–water (80:20, v/v) with 80 m*M* tetradecylammonium bromide. Capillary: 47 cm×50 μm I.D.; separation voltage: 10 kV; detection: 238 nm.

Table 1
Migration time repeatability and reproducibility for vitamin A palmitate, vitamin E acetate, and vitamin D_3

Compound	Migration time	
	Within-day variation R.S.D.% (n=6)	Between-day variation R.S.D.% (n=5)
Vitamin A palmitate	1.2	4.0
Vitamin E acetate	1.3	4.0
Vitamin D_3	1.3	4.2

with ethanol and 20 min with 1 *M* NaOH prior to beginning each day in order to effectively activate the silanol groups. Because tetradecylammonium bromide precipitated in alkaline media, the capillary was subsequently flushed for 25 min with water to remove the NaOH solution. In this way, plugging of the capillary was avoided when the first analysis was initiated by a 3-min rinse with the separation electrolyte. During the initial work, this activation procedure was carried out only once each day prior to the experiments. Although each analysis included a 3-min rinse with separation electrolyte, migration times varied considerably (Fig. 6) indicating a major problem in controlling the surface ionization of the fused-silica capillary. Thus, a short version of the activating program was tested, where the capillary was rinsed for 2 min with ethanol, 2 min with 1 *M* NaOH, and for 2 min with water prior to each analysis. In this way, the repeatability of migration times was significantly improved (Fig. 6). Although the time required for each analysis increased by 6 min, four separations were accomplished per hour.

3.4. Migration time and peak area stability

In order to evaluate the stability of the separation of the proposed CE system for hydrophobic nonionic compounds, both within-day and between-day variations of migration times and peak areas were investigated for the three vitamins. As illustrated in Table 1, migration times varied within 1.3% during the within-day experiment, while data varied within 4.2% for the between-day experiment. Similar results were obtained for peak area experiments Table 2. These results, which were obtained utilizing the above mentioned preactivation step between each analysis, indicated a very high separational stability of HIEKC with TDA^+ as the pseudostationary phase, and were almost comparable with data typically obtained by aqueous CE.

4. Conclusions

With vitamin A palmitate, vitamin E acetate, and vitamin D_3 as model compounds, the present work has demonstrated separation based on HIEKC in acetonitrile–water media with tetradecylammonium ions as the pseudostationary phase. The fat-soluble vitamins were analyzed with short separation times and with a very low consumption of organic solvents. In addition, the HIEKC separations were highly repeatable and reproducible. Thus, for the separation of mixtures of relatively low complexity, the present HIEKC setup may be very attractive. Additional work is in progress in order to further

Table 2
Peak area repeatability and reproducibility for vitamin A palmitate, vitamin E acetate, and vitamin D_3

Compound	Peak area	
	Within-day variation R.S.D.% (n=6)	Between-day variation R.S.D.% (n=5)
Vitamin A palmitate	1.7	3.1
Vitamin E acetate	1.2	4.2
Vitamin D_3	1.5	3.3

develop HIEKC for the separation of nonionic hydrophobic pharmaceuticals, and to extend the separation window.

Acknowledgements

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