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# Separation and simultaneous determination of water-soluble and fat-soluble vitamins by electrokinetic capillary chromatography ☆

M.M. Delgado-Zamarreño*, I. González-Maza, A. Sánchez-Pérez, R. Carabias-Martinez

*Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Ciencias Químicas, Universidad de Salamanca, 37008 Salamanca, Spain*

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**Abstract**

A comparative study of different surfactants, such as sodium dodecyl sulfate and bis(2-ethylhexyl) sodium sulfosuccinate (AOT), as pseudostationary phases was conducted in order to find the best conditions for the separation of the water- and fat-soluble vitamins by electrokinetic capillary chromatography. Separation was accomplished with AOT in a water–acetonitrile solution in the presence of boric acid–sodium borate buffer. A study of different variables was performed in order to obtain the best resolution and quantification of the vitamins: the variables explored were the pH and concentration of the buffer used, the percentage of acetonitrile, the concentration of surfactant and the applied voltage. Calibration curves and precision data were obtained for each analyte. Finally, a method to analyze these substances in pharmaceutical preparations is proposed. 

*Keywords:* Electrokinetic capillary chromatography; Buffer composition; Vitamins; Surfactants; Bis(2-ethylhexyl) sodium sulfosuccinate

## 1. Introduction

Capillary electrophoresis (CE) has been widely used in the past decade for the separation and determination of many substances of biological importance. Indeed, in some fields, CE is currently displacing high-performance liquid chromatography (HPLC) owing to is speed, high resolution and low cost. However, some of the drawbacks of CE include its rather poor concentration sensitivity and, sometimes, the impossibility of applying the method developed for standards to real samples. The aim of the present work was to study and optimize the separation of fat- and water-soluble vitamins and apply the method developed to their determination in pharmaceutical products. The water solubilities and the chemical characteristics of vitamins are very different, including their acid–base behavior.

The literature contains several references in which capillary zone electrophoresis (CZE) is used. Ward et al. [1] proposed a rapid CE method for the determination of total niacin in concentrated yeast spreads. After a complicated isolation of nicotinic

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*Corresponding author. Tel.: +34-923-294-483; fax: +34-923-294-574.

*E-mail address:* mdz@gugu.usal.es (M.M. Delgado-Zamarreño).

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acid from the food matrix, which included autoclaving the sample in aqueous calcium hydroxide, solid-phase extraction and cation-exchange chromatography, nicotinic acid was determined by CZE in a sodium tetraborate orthophosphate buffer at pH 9.2. Schiewe et al. [2] applied CZE to the determination of ascorbic acid, thiamine, nicotinamide, nicotinic acid and biotin in a simple run in vitamin mixtures (pharmaceutical formulations and also native citrus and fruit beverages). In the case of ascorbic acid, the addition of L-cysteine as an antioxidant was necessary for reliable results to be obtained. Using CZE with a high-sensitive cell, Mrestani and Neubert [3] determined thiamine in urine, plasma and saliva, improving the limit of detection by approximately ninefold as compared with standard capillaries. Fotsing et al. [4] described a method for the analysis of thiamine, nicotinamide, riboflavine, pyridoxine, ascorbic acid and pantothenic acid—all water-soluble vitamins—in a pharmaceutical formulation using CZE in a borax buffer, pH 8.5. The presence of cyanocobalamine interfered with the detection of nicotinamide and micellar electrokinetic chromatography (MEKC) was necessary.

However, most researchers have used MEKC to accomplish the separation of both neutral and ionic analytes, sodium dodecyl sulfate (SDS) being the pseudo-stationary phase most used. This mode of CE has been applied to resolve samples of water-soluble vitamins and fat-soluble vitamins. Blanco Gomis et al. [5] determined seven water-soluble vitamins in pharmaceutical formulations. Vidal-Valverde and Diaz-Pollan [6,7] proposed a method for the determination of thiamine in milk and meat; both samples had to be treated by enzymatic hydrolysis and purification of the extracts prior to MEKC. Boonkerd et al. [8] determined group B vitamins in different pharmaceutical products using CZE, MEKC and HPLC. The three techniques afforded similar results. The best results were obtained when paracetamol was used as internal standard. A few years earlier, Fujiwara et al. [9] determined thiamine, riboflavine, nicotinamide, pyridoxine and cyanocobalamine using MEKC, with ethyl aminobenzoate as internal standard.

Different authors have investigated the resolution of retinoids (retinol, retinal, retinoic acid, retynil acetate, retynil palmitate). In some cases, separation was carried out using MEKC with SDS in the presence of Brij 35 as the pseudostationary phase in a Tris buffer and 35% of acetonitrile [10]. In other cases, SDS and bile salts such as sodium cholate [11] or sodium deoxycholate [12] have been employed for the separation of liposoluble molecules. Pedersen-Bjergaard et al. [13] carried out the separation of retynil palmitate, tocopherol acetate, and vitamin $D_3$ in an acetonitrile–water (80:20, v/v) medium with tetradecylammonium bromide as pseudostationary phase.

Few publications describe the separation of water- and fat-soluble vitamins by CE. Using microemulsion electrokinetic chromatography (MEEKC), Boso et al. [14] achieved the separation of these analytes. As the separation system, these authors used a microemulsion of heptane or another similar solvent in solutions with surfactants. Using the same procedure, Altria [15] described the separation of vitamins A and D and water-soluble vitamins. Pedersen-Bjergaard et al. [16] also used MEEKC for the resolution and determination of fat-soluble vitamins in pharmaceutical products, modifying the microemulsion with 2-propanol to improve resolution.

Here, a solution of bis(2-ethylhexyl) sodium sulfosuccinate (AOT) surfactant in acetonitrile–water solution was used for the separation of the vitamins. The analytes studied were $B_1$, $B_2$, $B_6$, and H as water-soluble and $K_3$, A and E as fat-soluble vitamins. AOT has been previously studied by the authors [17] as the pseudostationary phase in electrokinetic chromatography for the resolution of mixtures of synthetic phenolic food antioxidants. This study compares the behavior of SDS and AOT as pseudophases in the separation of water- and fat-soluble vitamins by MEKC and demonstrates its use to analyze pharmaceutical products.

## 2. Experimental

### *2.1. Apparatus*

Electrokinetic chromatography was performed on a P/ACE System 2000 capillary electrophoresis apparatus equipped with a UV detector, permitting measurements at 200, 214, 254 and 280 nm (Beckman, Fullerton, CA, USA). The fused-silica capillary

used was 57 cm (50 cm to the detector)×75 μm I.D. (Supelco, Bellefonte, PA, USA). Data were recorded on a computer that uses the System Gold software to evaluate the electropherograms. To prepare the samples of pharmaceutical products, a centrifuge (Kokusan Enshinki, Tokyo, Japan) was used.

### 2.2. Reagents

Biotin (vitamin H), DL-α-tocopherol (vitamin E), thiamine hydrochloride (vitamin $B_1$ hydrochloride), riboflavine (vitamin $B_2$), pyridoxine hydrochloride (vitamin $B_6$ hydrochloride), retinol (vitamin A) and the surfactant, SDS, were supplied by Fluka (Alcobendas, Madrid, Spain). Menadione (vitamin $K_3$) was from Sigma (Alcobendas, Madrid, Spain). AOT surfactant was supplied by Aldrich (Alcobendas, Madrid, Spain). Methanol was from BDH (Poole, UK), acetonitrile from Merck (Barcelona, Spain); both were of HPLC grade. The other reagents were supplied by Panreac (Barcelona, Spain) and were of analytical-reagent grade.

### 2.3. Procedure

A solution of water-soluble vitamins was prepared in water at an approximate concentration of $10^{-3}$ *M*. Another solution of liposoluble vitamins in methanol was prepared at the same concentration. In each case, 1-ml aliquots of these solutions were diluted to 10 ml with the separation solution and injected under pressure into the capillary over 5 s. The separation conditions were varied in the study corresponding to each variable.

Between each run, the capillary was rinsed for 2 min with 0.1 *M* sodium hydroxide and a further 2 min with the buffer solution. A potential of 24 kV was applied to accomplish separation and detection was performed spectrophotometrically at 200 nm. In all experiments, temperature was maintained at 25 °C.

The treatment of real samples consisted of dissolving the commercial vitamin tablets with buffer solution. This solution was centrifuged at 3200 rpm, equivalent to an acceleration of 1835 *g* for the centrifuge used. After centrifugation, the sample was filtered through a 0.45-μm pore-size nylon membrane. When necessary, the pH of the sample was adjusted prior to injection.

## 3. Results and discussion

### 3.1. Study of separation

#### 3.1.1. SDS

Since SDS is the most widely used pseudostationary phase in CE, the SDS concentration, pH and buffer concentration were varied in order to resolve vitamins in the sample. The results obtained, under the best conditions, show that vitamins $B_6$ and H comigrate and the peaks of vitamins E and $B_1$ were broad and overlapped (Fig. 1). The addition of cyclodextrins and organic solvents ($CH_3OH$, $CH_3CN$), did not improve resolution.

#### 3.1.2. AOT

AOT is an anionic surfactant widely used as a model of organized systems and biological membranes. Its hydrophilic and lipophilic properties are very similar, which is one of the reasons why AOT is a vesicle-forming surfactant. The solubility of AOT in water is quite low but can be increased by adding low percentages of organic solvents. Acetonitrile is usually the best choice because its effects on the baseline noise and the electroosmotic flow are not very strong.

Several solutions containing 20 m*M* AOT and different percentages of acetonitrile (5–25%) were

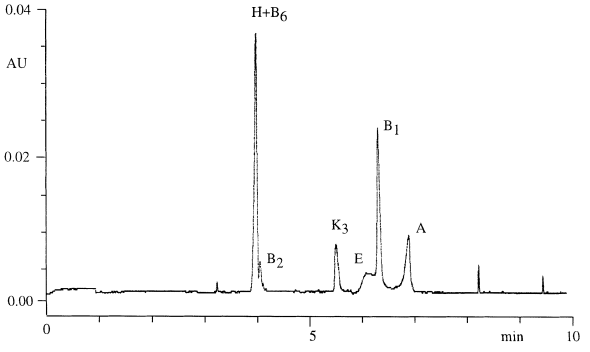


Fig. 1. Electropherograms obtained using the following conditions: injection under pressure (5 s); applied voltage 24 kV; separation buffer, 25 m*M* boric acid–sodium borate (pH 9.2), 30 m*M* SDS.

prepared. On increasing the concentration of acetonitrile, resolution improved, mainly for vitamins A and E. The best separation was achieved with 20% acetonitrile, but vitamins H and $B_6$ comigrated.

Next, the concentration of AOT was varied from 5 to 60 m*M*. From this study, two main results were obtained: the peaks of vitamins H and $B_6$ always overlapped, regardless of the AOT concentration, and vitamins A and E were separated when the AOT concentration was higher than 15 m*M*. When the AOT concentration was higher than 30 m*M*, the peaks of vitamins $K_3$ and $B_6$ overlapped. The best resolution (except for H and $B_6$) was achieved when the concentration of AOT (Fig. 2) was 25–30 m*M* and the run time was relatively short.

After the surfactant concentration had been optimized, 30 m*M* AOT solutions with different boric acid–borate buffer concentrations (10, 20, 30, 40 and 60 m*M*) were prepared. For values higher than 20 m*M*, resolution was appropriate but the migration times were long. Consequently, a concentration of 20 m*M* was selected because this afforded good resolution, short analysis times and low electrophoretic currents.

Finally, the pH values of the solutions were adjusted to 5.4, 6.8, 7.8, 8.2, 8.6, 9.0, 9.5, 9.8, 10.2 and 11.1 in order to optimize the resolution of the analytes. At low pH values, the results were not reproducible: the peaks were broad and poorly resolved. Between pH 7.8 and 8.6, all vitamins were separated, even H and $B_6$. The peaks of these vitamins overlapped again when pH values were higher than 8.6. Also, the peaks of vitamins A and E overlapped when the pH was higher than 9.8. At pH 11, peak resolution became progressively worse. The optimum pH for the separation was around 8.4.

Cyclodextrins, methanol or urea were added to the background electrolyte in an attempt to improve resolution and peak shape. However, only the addition of 2% methanol afforded a slight increase in resolution, stabilized the baseline noise and slightly increased the migration time window.

Fig. 3 shows an electropherogram obtained for a sample of standards under the optimum conditions: 20% acetonitrile, 2% methanol, 30 m*M* of AOT, 20 m*M* of boric acid–borate buffer and pH adjusted to 8.4. A good separation of the vitamins studied was obtained except for $B_2$ which comigrates with a system peak.

### 3.2. Analytical characteristics

With the optimized system, the electropherograms corresponding to different solutions containing the vitamins studied in the $1\cdot10^{-5}$–$1\cdot10^{-3}$ *M* range were recorded. The normalized areas of the vitamin peaks were plotted against their concentrations, yielding straight lines whose equations are shown in Table 1. Good linear correlation coefficients were obtained

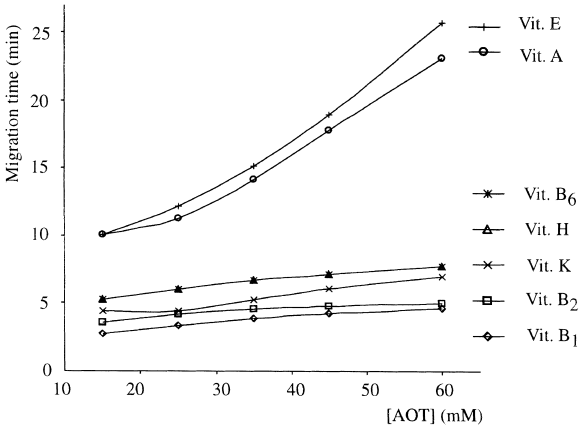


Fig. 2. Influence of AOT surfactant concentration on migration times. Conditions: acetonitrile–25 m*M* aqueous borate buffer (20:80, v/v), pH 9.8.

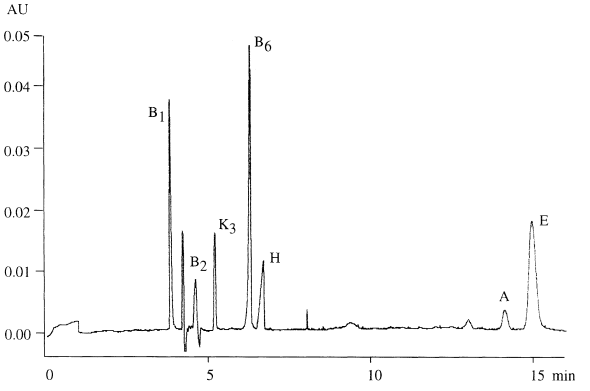


Fig. 3. Electropherogram of a standard mixture obtained using the optimal conditions: 25 m*M* AOT as pseudostationary phase, acetonitrile–25 m*M* aqueous borate buffer (20:80, v/v), pH 8.4 with 2% methanol. Separation voltage, 24 kV, pressure injection, 5 s.

Table 1
Analytical characteristics: calibration and precision

| Vitamin | Intercept | Slope | $R^2$ | Range ($M$) | RSD[a] (%) | RSD[b] (%) |
|---|---|---|---|---|---|---|
| $B_1$ | −1.4±2.1 | (2.6±0.2)·$10^4$ | 0.998 | $5\cdot10^{-5}$–$8\cdot10^{-4}$ | 1.1 | 7.5 |
| $B_2$ | −0.2±0.7 | (3.7±0.2)·$10^4$ | 0.997 | $1\cdot10^{-5}$–$1\cdot10^{-3}$ | 1.3 | 9.1 |
| $B_6$ | −0.2±0.3 | (5.8±0.1)·$10^4$ | 0.999 | $2\cdot10^{-5}$–$8\cdot10^{-4}$ | 1.2 | 12.2 |
| $K_3$ | −0.1±0.7 | (4.9±0.2)·$10^4$ | 0.997 | $2\cdot10^{-5}$–$8\cdot10^{-4}$ | 0.5 | 8.9 |
| A | −0.1±0.3 | (1.65±0.08)·$10^4$ | 0.997 | $2\cdot10^{-5}$–$8\cdot10^{-4}$ | 2.7 | 5.7 |
| E | −2.4±2.1 | (8.5±0.5)·$10^4$ | 0.996 | $2.5\cdot10^{-5}$–$1\cdot10^{-3}$ | 1.8 | 11.5 |
| H | 0.1±0.4 | (7.8±0.2)·$10^3$ | 0.999 | $5\cdot10^{-5}$–$3\cdot10^{-3}$ | 2.1 | 9.1 |

[a] Day-to-day relative standard deviation for migration times ($n$=10).
[b] Day-to-day relative standard deviation for peak areas ($n$=10).

for all the vitamins. Based on the inter-day relative standard deviations of the migration times and peak areas, the reproducibility of the separation and quantification of the analytes was good.

### *3.3. Analysis of vitamin tablet samples*

In CE, some organic substances including pharmaceutical excipients might adsorb on the wall of the fused-silica capillary and change the electroosmotic flow as described by Fotsing et al. [18] in the analysis of water-soluble vitamins in pharmaceutical products.

In order to decrease excipient adsorption and find a simple treatment for the samples, several assays were carried out with and without centrifugation and filtration of the samples. Both centrifugation and filtration were necessary to minimize adsorption of the excipients; when only filtration was used adsorption on to the capillary wall was higher.

Methanol, water, and separation buffer were tested as solvents for the extraction of the analytes. The best results were obtained with the separation buffer because this dissolved both the liposoluble and hydrosoluble vitamins.

The proposed analytical procedure was as follows: vitamin tablets were ground and homogenized, 100–200 mg were weighed and dissolved in 5 ml of the separation buffer. The sample was then placed in a sonication bath for 5 min to aid extraction and dissolution of the analytes. Then, the sample was centrifuged and filtered through a 0.45 μm nylon membrane. Owing to the large amount of phosphates and carbonates present in the tablets, it is necessary to adjust the pH to 8.4 because the pH of the sample must be similar to that of run buffer solution. Finally, the solution was brought up to volume (10 ml) with separation buffer and injected into the electrophoresis system over 5 s. Table 2 shows the results obtained for two commercial vitamin tablets as compared with the levels stated by the manufacturer.

## 4. Conclusions

The results of the present work indicate that it is possible to separate, with good resolution, simultaneously fat- and water-soluble vitamins by electrokinetic chromatography with a simple background electrolyte composed of sodium borate–boric acid and AOT as the pseudostationary phase. Using SDS, total separation was not achieved. It should be stressed that the interactions between vitamins and pseudostationary phases were different, as can be observed in the change in the migration order when

Table 2
Analysis of a commercial multivitamin tablet using the proposed procedure

| | Vitamin $B_1$ | Vitamin $B_2$ | Vitamin $B_6$ | Vitamin E | Vitamin A |
|---|---|---|---|---|---|
| Labeled (mg) | 1.4 | 1.6 | 2 | 10 | 0.5 |
| Found (mg) | 1.71±0.4 | 1.64±0.3 | 2.75±0.6 | 12.5±2 | – |
| % of labeled amount | 122.1 | 102.5 | 136.5 | 125.0 | |

AOT was used. This methodology can be applied to determine vitamins in pharmaceutical products.

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