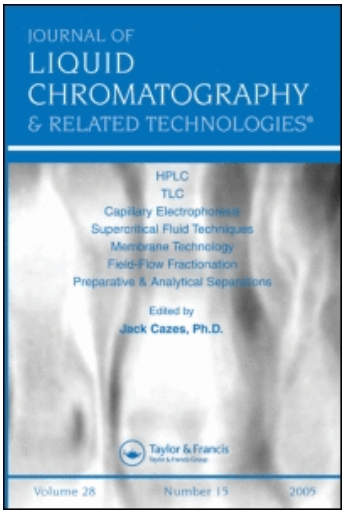

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Abstract: The separation of six water-soluble vitamins (e.g., B_1—thiamine, B_2—riboflavin, B_3—nicotinic acid, B_6—pyridoxine, B_{12}—cyanocobalamin, and C—ascorbic acid) was investigated using capillary electrophoresis. Initially, the most important variables in micellar electrokinetic capillary chromatography were established, such as: background electrolyte pH; surfactant concentration; detection wavelength; applied voltage, and amount of organic modifier. The solutes were separated within 8 min, with excellent efficiency ($3 \cdot 10^5$ theoretical plates) and resolution, which means great advantage towards the analysis of real samples. Two pharmaceutical samples were analyzed using the optimized method, which was shown to be adequate for this purpose.

Keywords: Vitamins, Capillary electrophoresis, MECC parameters, Pharmaceutical samples

INTRODUCTION

Vitamins are organic food substances that are only found in plants and animals. With few exceptions the human body can not synthesize them;

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therefore, they must be consumed in diet or food supplements. Nowadays, the use of food supplements has increased and extensive research on disease treatments (such as cancer[1–6] depression,[7] neurodegenerative,[8] and chronic diseases[9]) with vitamins has been carried out. Their manufacture demands fast, efficient, reliable, reproducible, and simple analysis. For this reason, the interest in new methods to determine these analytes in various matrices, such as capillary electrophoresis (CE), has increased lately. Vitamins may be classified as water-soluble or fat-soluble, according to their hydrophobicities.[10] In this paper the analysis of water-soluble vitamins will be presented. Water soluble vitamin structures, as well as their sources and use, are described elsewhere.[11]

Capillary electrophoresis (CE) has expanded greatly in recent years due to many advantageous features, such as: low consumption of background electrolyte (BGE) and sample, reduced sample preparation, fast analysis, high efficiency, and high resolution. Among the several CE modes, capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) are the most used methods. MECC development has presented a great interest since CZE can not separate neutral solutes; this is because they migrate together with the electroosmotic flow (EOF). Since Terabe et al.[12] started investigation on MECC, neutral compounds could also be separated in a single run, due to the presence of micelles. Besides, ionic solutes are better resolved through this technique due to:

a) the negative charge some micelles have and the number of negative charged groups in the solute;
b) the distribution of the solute between the micellar and aqueous phases (hydrophobicity); and
c) ionic pair formation between the cationic group of the solute and the polar group of the anionic surfactant.

Basically, the mechanism of separation consists of distribution of dissolved compounds between two phases (micellar phase = pseudo stationary phase, and surrounding phase = mobile phase) migrating at different velocities. Consequently, neutral solutes with differences in the distribution constant K_{MS} can be separated in a manner similar to that of a chromatographic separation.[13,14] Micelles are formed by the addition of a surfactant in an amount larger than the critical micelle concentration (CMC). Sodium dodecyl sulfate (SDS) has been the most used anionic surfactant in MECC. SDS has gained the same status as octadecyl silane in high performance liquid chromatography (HPLC),[15] although the use of polymers has also been focused for the search for more versatile micelles.[16]

The use of MECC is more advantageous than CZE towards analysis of water-soluble vitamins because these analytes possess different neutrality. Vitamins B have been separated by CZE and MECC, since these techniques are considered a good alternative to HPLC.[17,18] The determination of

ascorbic acid in juices and beverages,[19–22] as well as in body fluids,[23,24] by CE has been extensively used since it offers short analysis time and is less expensive than HPLC; analysis of water-soluble vitamins in food and beverages by CE has been reviewed by Trenerry.[25] Separation of samples containing different water-soluble vitamins has been widely carried out by MECC[26–29] in pharmaceutical samples,[30–35] and commercial capsules[36,37] are very commonly analyzed through this technique. Usually, ultra violet detectors are used in CE; nevertheless, electrochemical[38,39] and laser-excited fluorescence[40] detection have already been employed for the determination of vitamin B_6, indicating a higher sensitivity of detection.

The separation of six water-soluble vitamins in commercial pharmaceutical samples by MECC, with UV detection, will be shown in this paper. Several parameters have been investigated according to their influence on peak intensity, migration time, and efficiency, namely wavelength detection; applied voltage; surfactant concentration; pH; and amount of organic modifier.

EXPERIMENTAL

Chemicals and Reagents

Boric acid (Vetec, Rio de Janeiro, RJ, Brazil), was used to prepare a 20 mM background electrolyte (BGE). pH of basic BGEs was adjusted with a 4 M sodium hydroxide (Synth, São Paulo, SP, Brazil) solution; pH of acidic BGE's was adjusted with 1 M hydrochloric acid (Mallinckrodt, Mexico, Mexico) solution. Sodium dodecyl sulfate (Polysciences, Warrington, USA), was dissolved in the BGE until the chosen concentration. Acetone (Mallinckrodt, Mexico, Mexico) was used as EOF marker. Methanol (Mallinckrodt, Kentucky, PA, USA), was used as the organic modifier.

Vitamin analytical standards were acquired in a neighborhood manipulation pharmacy; vitamin C was purchased from Reagen (Rio de Janeiro, RJ, Brazil). Standard solutions were prepared daily in volumetric flasks protected from light (due to solute instability). The solutions were prepared in the following concentrations: B_1—100 mg/L; B_2—50 mg/L; B_3—50 mg/L; B_6—50 mg/L; B_{12}—100 mg/L; C—100 mg/L. Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all solutions.

Apparatus

Capillary electrophoresis experiments were carried out either in a Quanta 4000 CE System (Waters, Milford, MA, USA) or in a HP3DCE system (Agilent Technologies, Waldbron, Germany). The UV detection was done

on-column in both of them. In the former, a fixed wavelength detector was used, which was set at 214 nm; the separation was carried out in a 52.5 cm effective length (L_T) and 75 μm internal diameter (i.d.) fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). In the second one, a diode array detector was used and the separation was performed in a 50 cm effective length and 50 μm i.d. fused silica capillary.

Electrophoretic Procedure

Capillary conditioning was carried out daily by flushing with the following solutions: 1 M sodium hydroxide for 5 min; 0.1 M sodium hydroxide for 5 min; BGE for 15 min. Between electrophoretic runs, the capillary was flushed only with fresh BGE for 2 min. EOF marker was added to all standard solutions which were hydrodynamically injected for 10 s. Commercial samples were hydrodynamically injected for 3 s.

Commercial Sample Preparation

The solid sample (a tablet of vitamin C) was ground and 0.800 g of the powder was dissolved in 20 mL of ultrapurified water, yielding a 400 mg/L solution of

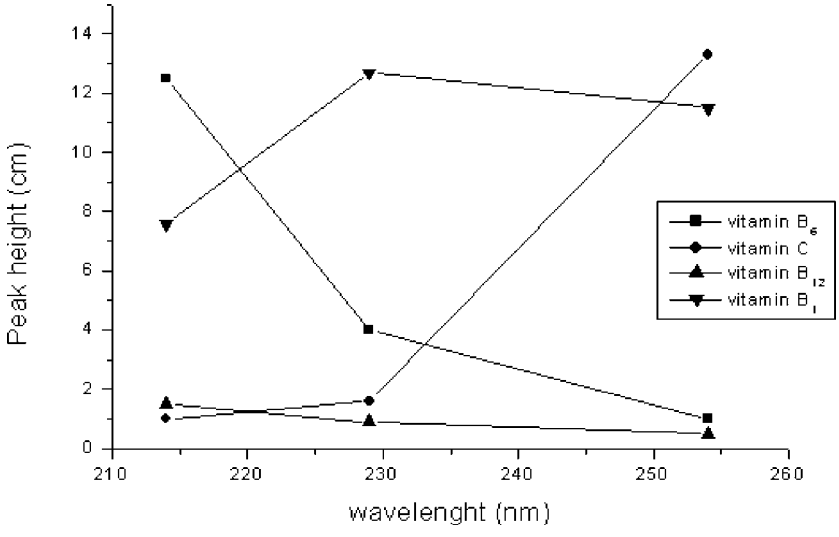

Figure 1. Detection wavelength influence on peak height of vitamins B_1, B_6, B_{12}, and C analyzed by MECC. Analysis conditions: 20 mM borate buffer pH 9.0; [SDS] = 50 mM; applied voltage: 25 kV; injection time: 10 s; L_t = 52.5 cm; i.d. = 75 μm. Analytes concentrations: B_1—100 mg/L, B_6—50 mg/L, B_{12}—100 mg/L, C—100 mg/L.

the vitamin. The solution was filtered on a disk membrane filter of 0.45 μm pore size (Lida, Kenosha, WI, USA). The liquid commercial sample was only diluted to 50% with ultrapurified water.

RESULTS AND DISCUSSION

Figure 1 shows the differences in peak intensities, according to detection wavelength, of a four water-soluble vitamin separation. At 214 nm, vitamins B_6 and B_1 show the highest peaks, in the same run. Although vitamins B_{12} and C present short peaks at this detection wavelength, this filter was chosen to be used at the detector when the Quanta 4000 CE System carried out the electrophoretic runs, because that was the best compromise for detection of all solutes.

To define the best separation voltage to be applied in the system, its influence on separation velocity was investigated (Figure 2). Although the fastest run is achieved with 30 kV, 25 kV also presents optimum running conditions. As presented in Figure 3, at 25 kV, the separation of three water-soluble vitamins is very well resolved, the efficiency is high, and the separation is fast (around 5.5 min). The Joule effect (capillary heating) is minimal when the voltage decreases and it is unlikely to happen during the

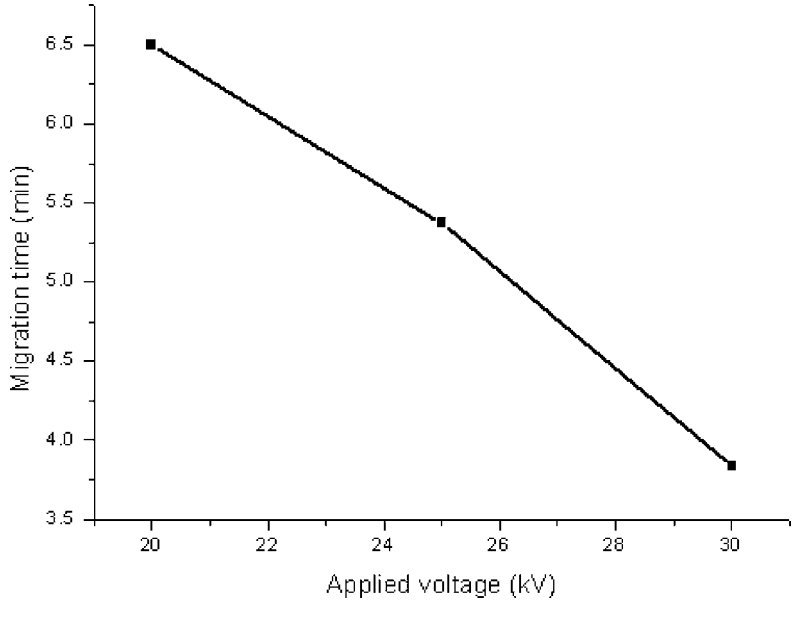

Figure 2. Influence of applied voltage on migration time in the analysis of vitamins B_1, B_6, B_{12}, and C. Analysis conditions are the same as presented in Figure 1 except for $\lambda = 214$ nm.

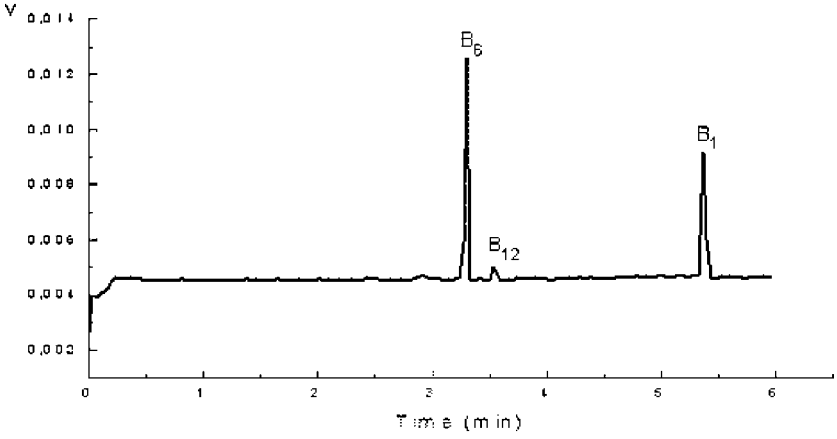

Figure 3. Electropherogram (MECC) illustrating the separation of vitamins B_6, B_{12}, and B_1 at 25 kV. Analysis conditions: 20 mM borate buffer, pH 8.0 containing 30 mM SDS; detection wavelength: 214 nm. Other conditions are the same as in Figure 1.

separation of unstable analytes (such as vitamins). Thus, 25 kV was chosen to be applied for the analysis of vitamins.

The amount of SDS, the selected surfactant for this study, in the BGE and its effect on analysis time was investigated (Figure 4). When solutes are

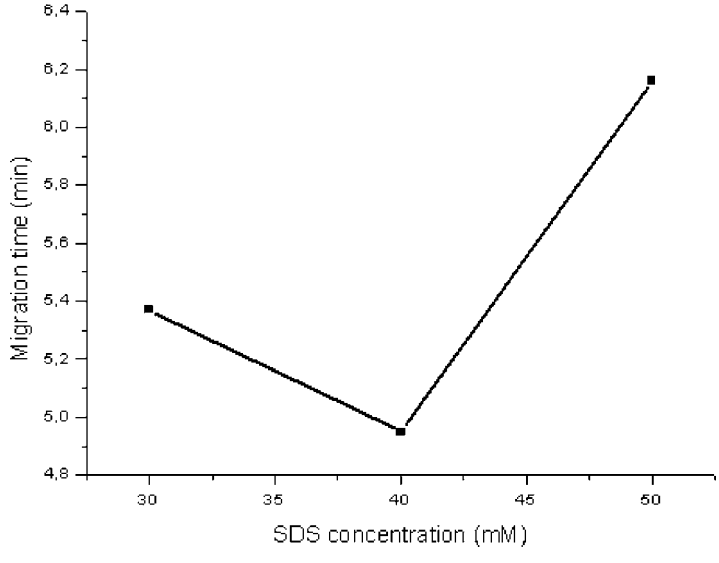

Figure 4. Migration time variation towards surfactant concentration on the analysis of vitamins B_1, B_6, B_{12}, and C. Analysis conditions are the same as in Figure 2 but applied voltage = 25 kV.

electrically neutral, the migration time is proportional to SDS concentration. Indeed, if migration time is independent of SDS concentration, the solute is totally excluded from the micelle; on the other hand, if migration time variation is observed while the amount of SDS is changed, it is concluded that micelles and solutes are interacting. Therefore, vitamins do possess ionizable groups, besides hydrophobic ones, because changes in surfactant concentration modify their migration times.[13]

The migration time has decreased, changing from 30 mM to 40 mM of SDS concentration due to temperature influence on solute mobility, as the system (Quanta 4000 CE System) used in this experiment has no temperature control. However, an increase in running time has been noted, as well as separation resolution (Figure 5), towards SDS concentration increase. When vitamin C was added to the analytical standard mixture, a co-migration with vitamin B_{12} occurred. Thus, other parameters had to be optimized to improve separation conditions. For this reason, 50 mM SDS was used in the succeeding investigations.

Among several parameters, migration time and efficiency are substantially affected by pH changes in the buffer. According to Equation (1), the efficiency (N) obtained at each studied pH was calculated and plotted, as shown in Figure 6.

$$N = 16\left(\frac{t_m}{W}\right)^2 \tag{1}$$

Figure 7 shows the migration velocity increase of some solutes until pH 8.0. Accordingly, the electroosmotic flow (EOF) also increases with BGE pH, due to ionization of the inner capillary wall's silanol groups. From this pH on, there is no velocity enhancement due to maximum ionization of these groups. Vitamin C is a very unstable compound. At pH 10, it is not

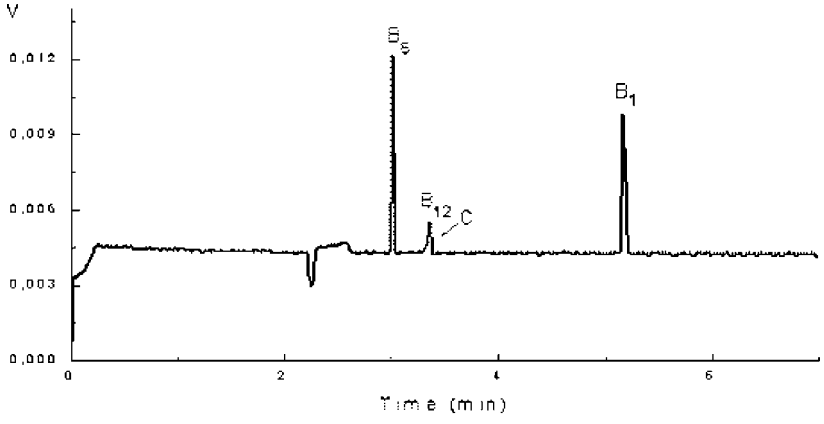

Figure 5. Electropherogram (MECC) illustrating the separation of vitamins B_6, B_{12}, C, and B_1 with 50 mM of SDS in the BGE. Other conditions are the same as Figure 2.

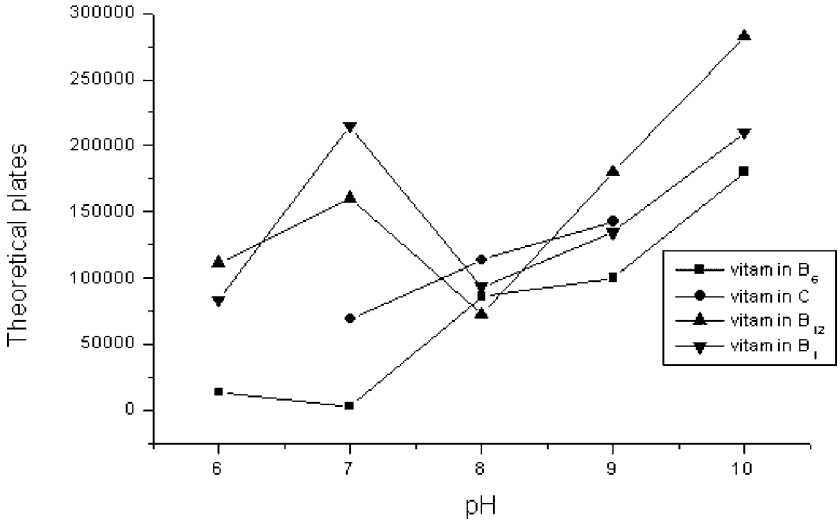

Figure 6. Effect of BGE pH on the separation efficiency of vitamins B_1, B_6, B_{12}, and C peaks using MECC. Analysis conditions are the same as in Figure 5 but with different BGE acidities.

possible to observe this solute because of its easy oxidation; the higher the pH, the faster its decomposition.[41] Due to high efficiency and different solute migration times, a BGE of pH 9.0 was selected for the subsequent runs.

Separation of vitamins at pH 9.0 is shown in Figure 8. The order of solute migration may be explained by their molecular structures, which are described elsewhere.[10] Pyridoxine (B_6) has three hydroxyl groups, which means great affinity for the aqueous phase; for this reason, the affinity for micelles is decreased and this vitamin is the first one to be detected. Ascorbic acid (C) presents carbonyl and hydroxyl groups (hydrophilics), but intramolecular interactions diminish its affinity for water. Cyanocobalamin (B_{12}) contains a metal (Co^{2+}), but many bulky groups surround it; consequently, the mobility of the solute is low, as well as its affinity for water. Thiamine (B_1) presents cationic groups which form ion pairs with the anionic tails of micelles. This means that its affinity for the micellar phase is higher than the other vitamins and it is the last one to be detected.

The introduction of an organic modifier to the electrophoretic medium influences the mobility and pK_a's of solutes, as well as the EOF, thereby increasing resolution. The higher the amount of modifier, the slower the velocity of EOF, due to higher buffer viscosity and decrease of zeta potential of the capillary silanol groups.[42,43]

In this paper, different amounts of methanol were added to study its influence on the separation of water-soluble vitamins (Figure 9). Amounts higher than 20% of organic modifier were not added to the buffer solution,

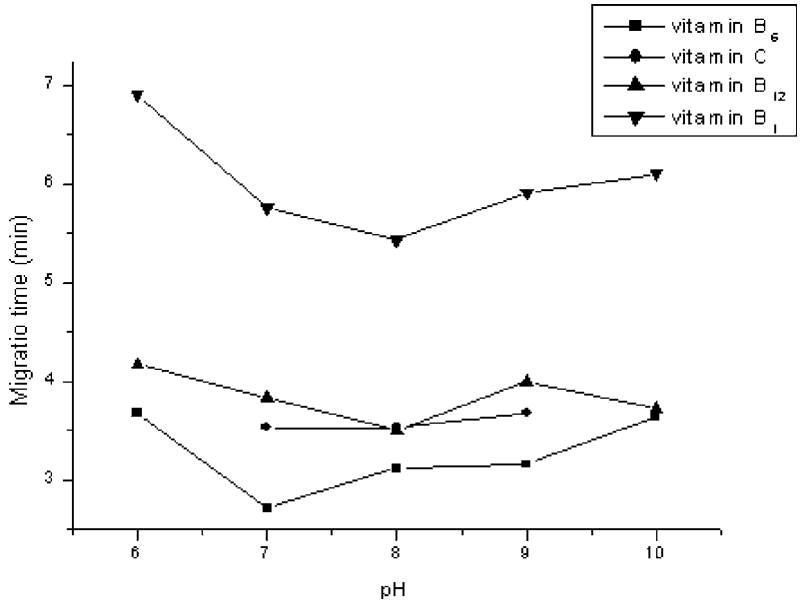

Figure 7. Buffer pH influence on vitamins B_1, B_6, B_{12}, and C migration time. Analysis conditions are the same as in Figure 6.

due to its influence on micelle formation. It is noted that the percentage of organic modifier changes the order of migration of some solutes. This may happen according to different affinities of each one by the modifier. Therefore, solutes which had greater affinity for micelles before adding

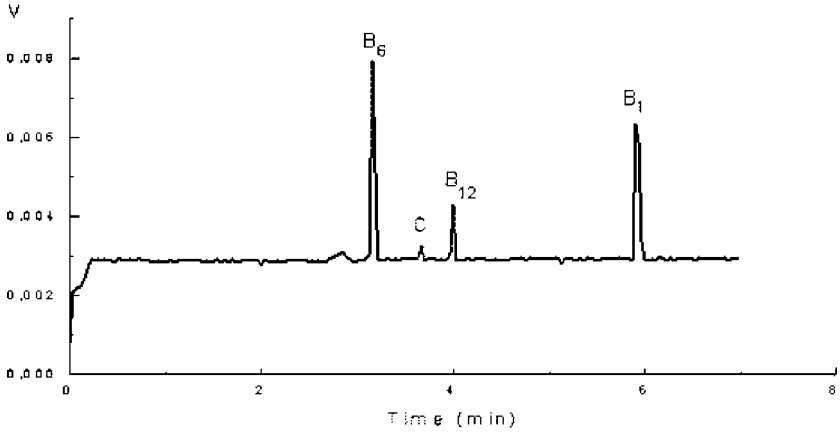

Figure 8. Separation of water-soluble vitamins B_1, B_6, B_{12}, and C at pH 9.0. Analysis conditions as in Figure 5.

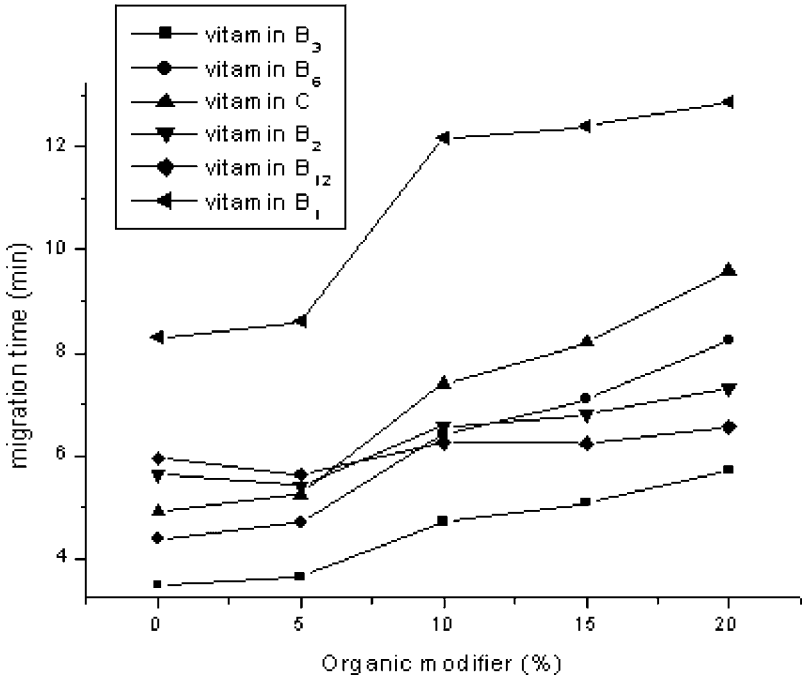

Figure 9. Dependence of migration time of water-soluble vitamins on the amount of organic modifier. Analysis conditions as in Figure 8.

methanol have the tendency to remain less in the micelles' interior because of the affinity for methanol in the electrolytic medium. When the HP3DCE system was used, the electropherograms' baselines presented a very low noise ratio (Figure 10). For this reason, small differences among solute migration times were observed, even when no organic modifier was introduced.

The migration order of vitamins B_2 and B_3 can also be predicted by their respective molecular structures. Vitamin B_3 presents the largest affinity for aqueous phase of all the water-soluble vitamins studied herein, as it is a simple acid (it bears only one carboxylic group), being the first solute to migrate. Vitamin B_2 has many polar groups, but the carbonic chains are bigger than those found in vitamins B_6 and C; thus it presents more affinity for micelles.

After the evaluation of the major experimental variables on the separation of the investigated vitamins, two pharmaceutical samples were analyzed through the optimized method, i.e., samples A and B. Four studied water-soluble vitamins could be identified in sample A (Figure 11): B_6, B_2, B_{12}, and B_1. According to this formulation listing, it does not contain vitamin B_3. The third peak of the electropherogram was unidentified, although

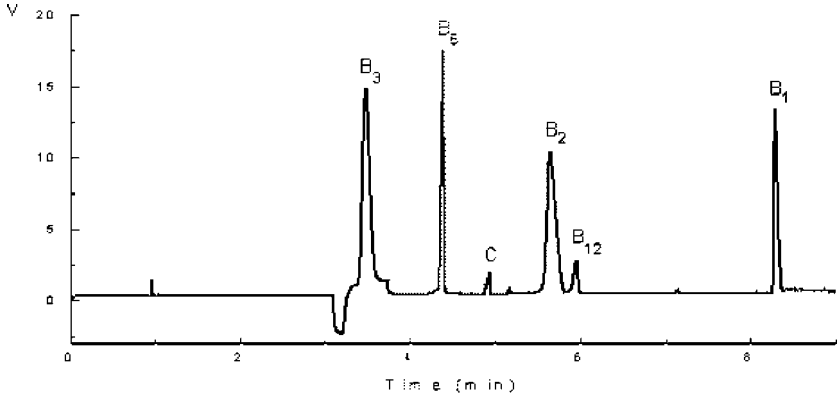

Figure 10. Electropherogram (MECC) illustrating the separation of six water-soluble vitamins with 0% of organic modifier in the BGE. Analysis conditions are the same as in Figure 8.

observation of its UV absorption spectrum indicates it is vitamin B_5 (calcium pantothenate); the last peak is also unidentified; it may be attributed to vitamin E, due to its high hydrophobicity. Sample A contains both vitamins, as indicated in its label, as well as vitamin C. However, vitamin C was not observed.

Sample B is a tablet which contains only ascorbic acid.[44] As shown in Figure 12, two peaks have been observed. However, according to migration time and UV absorption spectrum (data not presented), only the first one can be attributed to vitamin C.

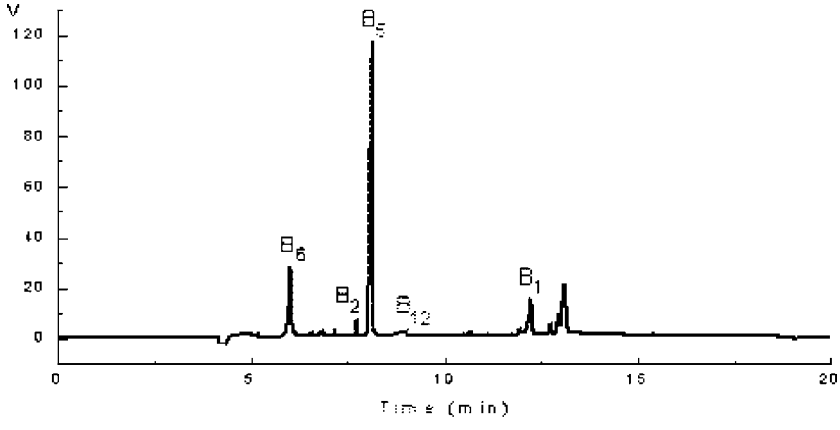

Figure 11. Electropherogram (MECC) illustrating the separation of water-soluble vitamins in pharmaceutical sample A. Analysis conditions are the same as Figure 8.

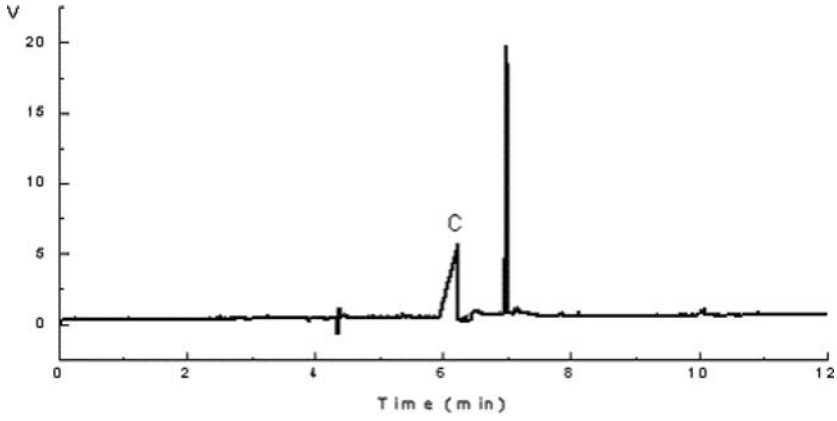

Figure 12. Electropherogram (MECC) illustrating the analysis of vitamin C in pharmaceutical sample B. Analysis conditions are the same as in Figure 8.

CONCLUSION

MECC proved to be a reliable method of analysis for vitamins in pharmaceutical samples. Fast separation time for the six analytes, with high efficiency ($3 \cdot 10^5$) and resolution, was achieved (about 8 min). This means great advantage for using this technique in water-soluble vitamins analysis, compared to conventional HPLC methods. The velocity of separation is very important in routine analysis (mainly in industry) and when unstable solutes are analyzed. This technique does not require extensive sample preparation, since MECC presents high efficiency and resolution.

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