Simultaneous Determination of Nine Acetylcholinesterase Inhibitors Using Micellar Electrokinetic Chromatography

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Abstract

MEKC was used for the separation of nine acetylcholinesterase inhibitors (AChEIs). AChEIs are an important group of drug compounds that are used medicinally to treat Alzheimer's disease and Myasthenia Gravis. At the time of the experiment, this is the first time that nine AChEIs are used simultaneously in a study. Several chromatographic parameters, such as buffer concentration, pH, surfactants and their concentration, background electrolyte composition, etc., were evaluated to optimize the separation. The optimum separation of the nine AChEIs was achieved in less than 15 min by using 12.5 mM Na_2HPO_4, 12.5 mM $Na_2B_4O_7$ and 20 mM SDS at pH 10, an applied voltage of 30 kV and a temperature of 25°C. The reproducibility of the method was also evaluated by computing the RSDs of the migration times and the areas of the nine analyte-peaks, and the migration time and the area of the peak that corresponds to rivastigmine added in the blood sample. The RSD values of the migration times and the peak areas were less than 2% and 6%, respectively, in most cases. The limits of detection and quantification were 0.5 µg/mL and 1.7 µg/mL, respectively. The MEKC method developed was applied to a real blood sample that was obtained from a patient who was not under any of this medication. The sample was spiked with rivastigmine in order to establish the ability of the method to separate the drug from other components that might exist in the blood sample.

Introduction

Alzheimer's disease (AD) is the most common form of degenerative dementia of human central nervous system. Clinically, AD is characterized by progressive impairments in memory and other cognitive domains. The neuropathological hallmarks of AD include neurotic plaques (1), neurofibrillary tangles (2) as well as the ε4 allele of apolipoprotein E (3). One of the earliest pathological events in AD is thought to be the degeneration of cholinergic neurons of the basal forebrain (4). The primary therapeutic approach to address the cognitive loss associated with AD has been that of a cholinergic replacement strategy. Myasthenia Gravis (MG) is an autoimmune disease characterized by fatigable muscle weakness. It is associated with autoantibodies that are bound to the acetylcholine receptors on the postsynaptic membrane at the neuromuscular junction (5). Acetylcholinesterase inhibitors (AChEIs) are also the main therapeutic approach for MG since these drugs inhibit the synaptic enzyme, acetylcholinesterase, which leads to an increase in acetylcholine at the post-synaptic membrane. So, AChEIs are considered an important group of drug compounds that can be medicinally used for the treatment of both diseases.

As far as could be ascertained, this is the first time that nine AChEIs have been separated simultaneously in a single run. In literature, the use of several analytical techniques is reported for the analysis of a single AChEI at a time, usually in biological fluids. Several methods have been reported for the determination of physostigmine and its metabolites in biological matrices and in pharmaceutical formulations. These methods include high-performance liquid chromatography (HPLC) (6–9) and capillary zone electrophoresis (CZE) (10). The determination of physostigmine and its metabolites in biological fluids has been reviewed by Zhao et al. (11). Pyridostigmine has been determined in pharmaceutical preparations by CZE (10) as well as by HPLC in rat plasma and urine (12). The method developed could be used to assess distribution of the parent compounds and metabolites in body tissues and fluids following real-life exposure (12). Galanthamine has also been determined by CZE in biological fluids and pharmaceutical formulations (13). It was observed that the addition of magnesium chloride has permitted a better separation of galanthamine from the other serum components. The determination of rivastigmine, a relatively new drug used for the treatment of AD, has not been widely reported in the literature. Liquid chromatography-tandem mass spectrometry (LC–MS–MS) (14–16) as well as gas chromatography-mass spectrometry (GC–MS) (17,18) assays are reported for the determination of rivastigmine and its metabolite. However, the LC–MS–MS method is now the preferred choice due to its faster chromatographic procedure and its higher sample throughput compared to the GC–MS method (16).

The purpose of this study is the development of a CE method for the simultaneous determination of nine AChEIs. Micellar electrokinetic chromatography (MEKC) is a powerful and efficient technique used for the separation of both neutral and

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charged compounds. Therefore, MEKC was chosen for the separation of AChEIs. Several chromatographic parameters such as the buffer concentration, the pH, the SDS concentration, the background electrolyte composition, as well as the injection time, the applied voltage, and the temperature were evaluated to optimize the separation. In addition, optimum conditions were applied to a real blood sample that was obtained from an AD patient who was not under any of this medication. The sample was spiked with rivastigmine so that the ability of the method to separate the drug compound from all the others components that might exist in the blood sample can be established. The chemical structures and the numerical designations of these substances are shown in Figure 1.

Experimental

Apparatus and conditions

All MEKC experiments were performed on an Agilent, G1600A Capillary Electrophoresis System equipped with an on-column diode-array detection system (Santa Clara, CA). Since the best signal-to-noise ratio was observed at 214 nm, this wavelength was used in this study. The instrument contains a 0-30-kV high voltage power supply. Data were collected using ChemStation software. Separations were carried out using bare fused-silica capillaries with a total length of 64 cm, an effective length of 55.5 cm and an inner diameter of 50 µm. New capillaries were rinsed with water for 5 min, 1 M sodium hydroxide for 1 h, again with water for 10 min and finally with the separation electrolyte for 30 min. Between injections, the capillary was conditioned by use of the buffer for 5 min. The samples were injected hydrodynamically towards the cathode. Unless stated otherwise, the capillary was thermostated to 25°C. The pH measurements were performed on a Hanna Instrument pH 211, Microprocessor pH (Ann Harbor, MI).

Reagents and chemicals

All pharmaceutical reference substances used in this study, except rivastigmine, were obtained from Sigma-Aldrich (Steinheim, Germany). The buffers used as background electrolytes were: sodium tetraborate decahydrate ($Na_2B_4O_7 \times 10\ H_2O$) (Fluka Biochemika, Munich, Germany), sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich), and tris(hydroxymethyl) aminoethane [$(OHCH_2)_3CNH_2$] (Scharlau Chemie S.A, Barcelona, Spain). Sodium Dodecyl Sulfate was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany). Acetonitrile and methanol were HPLC grade, and were purchased from Sigma-Aldrich. The pH of the BGE was adjusted using either sodium hydroxide or phosphoric acid. Before use, buffer solutions were filtered through a 0.45-µm syringe filter and sonicated for 5 min.

Sample preparation

The stock solutions (0.9 mg/mL) of AChEIs, except rivastigmine, were prepared by dissolving the appropriate amount of solid in methanol–water (50:50) and stored at 4°C. Then, a 10-µL aliquot of each analyte was mixed. The final concentration of each analyte in the mixture was ~0.1 mg/mL. Rivastigmine was extracted from the pharmaceutical pill Exelon according to the following procedure (19): The content of 10 capsules was emptied and homogenized. The powder was transferred into a flask with 60 mL methanol and was placed in an ultrasonic bath for 10 min. Then, the mixture was centrifuged at 1300 × *g* for 15 min, and the supernatant was isolated. Ten microliters of rivastigmine was mixed with the mixture of the other eight analytes. Prior to analysis, the whole blood sample was diluted 10-folds with the buffer solution (12.5 mM phosphate, 12.5 mM tetraborate, 20 mM SDS, pH 10).

Results and Discussion

As mentioned earlier, until now, no simultaneous determination of AChEIs has been reported. Thus, the aim of the study reported here was to provide an appropriate analytical method for the separation of nine AChEIs.

Separation using CZE

Initially, the simplest mode of CE, called CZE, was used. In this case, the capillary was filled only with the buffer solution (12.5 mM tetraborate, 12.5 mM phosphate, pH 10). No separation was achieved under these experimental conditions. Solutes eluted before or with the EOF. This suggests that some of the analytes are cationic, so they migrate toward cathode before the EOF, and some of them are neutral and coelute with the EOF. Thereby, a negatively charged surfactant must be used to reduce the mobility of the solutes and improve the separation. In MEKC, a

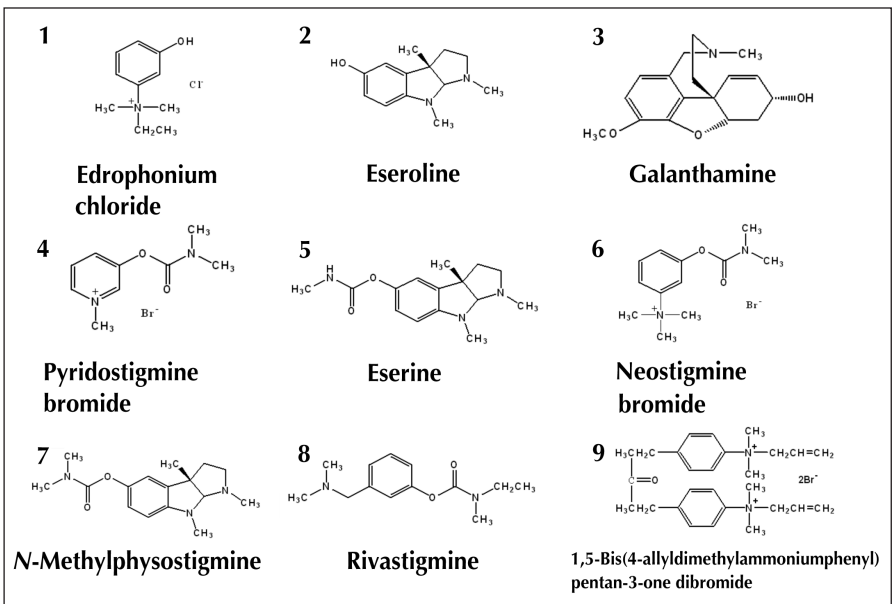

Figure 1. Chemical structures and numerical designations of the nine AChEIs.

pseudostationary phase is constituted by surfactants that are added in the running buffer. It can be used for the separation of both neutral and charged solutes.

MEKC method development

For the MEKC method development, the influence of different operating parameters such as buffer electrolyte concentration and pH, SDS concentration, injection time, applied voltage, and temperature were evaluated in order to optimize the MEKC separation of the AChEIs.

Effect of background electrolyte

The selection of the running buffer is extremely important for the success of any CE separation since it alters selectivity. The buffer systems phosphate, Tris–borate and phosphate–borate at various concentrations were used as background electrolytes to determine the one that provides the best separation of the nine AChEIs. The use of an organic modifier was considered not necessary because, in the case of ACN, resolution was poor, and in the case of MeOH, the total analysis time was long and the resolution was low. In each buffer system, the pH was adjusted at 10. When the buffer system Tris–borate was used, all the analytes eluted fast and the resolution was low (data is not shown). The use of phosphate buffer as background electrolyte resulted in good peak efficiencies and short analysis time, while the resolution of peaks 7 and 8 was low (R_s = 0.66). The borate–phosphate buffer provided the best results in regards to analysis time, efficiency and resolution. The concentration of the last buffer was varied in order to obtain the best possible separation of the analytes under study. As demonstrated in Figure 2, retention times of the analytes increased with increasing buffer concentration. The increase in migration time is due to a decrease in zeta potential. These longer migration times resulted in lower peak efficiencies (Table I). For total concentration of 30 mM, two different concentration ratios of borate–phosphate buffer were examined [20 mM tetraborate and 10 mM phosphate (Figure 2C) and 15 mM tetraborate and 15 mM phosphate (data is not shown)]. In both cases, analytes 3 and 4 coeluted. Taking into consideration the resolution, the peak efficiency, and the migration times, the equimolar mixture of phosphate and borate buffer with a total concentration of 25 mM (pH 10) was used to further optimize separation conditions.

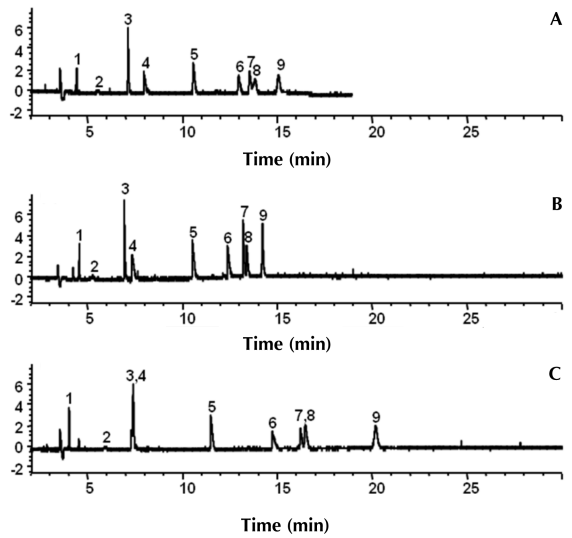

Figure 2. Effect of buffer concentration on the separation of the nine AChEIs (pH 10): (A) 12.5 mM tetraborate and 5 mM phospate, (B) 12.5 mM tetraborate and 12.5 mM phosphate, and (C) 20 mM tetraborate and 10 mM phosphate. Conditions: SDS, 20 mM; pressure injection, 30 mbar for 1 s; applied voltage, 30 kV; temperature, 25°C; fused-silica capillary, 64 cm (55.5 cm effective length) × 50 µm i.d.; detection, 214 nm.

Effect of pH

The pH of a buffer system is a critical parameter for the development of a MEKC method because ionic analytes demonstrate remarkably different affinity with respect to the ionic micelles, depending on the ionic charge status (20). The pH has also a remarkable effect on the EOF generated in a fused-silica capillary. In MEKC, buffers with high pH values are typically used in order to maintain a reasonable EOF and ensure migration direction (21).

The effect of the pH on the resolution and the analysis time was investigated over a range of 8–10 using the optimum buffer system 12.5 mM phosphate, 12.5 mM borate, and 20 mM SDS. As depicted in Figure 3, at a constant ionic strength, migration times decreased with increasing the pH due to a change in the EOF. EOF at higher pH values becomes faster, which, in turn, reduces the migration times. As a consequence of the latter, higher efficiencies were obtained, particularly for the last three peaks (Table II). In addition, at higher pH values, an increase in resolution between the peaks 7 and 8 was observed (Table II). Based on these results, pH 10 was used to further optimize the conditions for the separation of the AChEIs.

It is interesting to note that the elution order of some analytes changed when different pH values were used. For example, while

Table I. Efficiencies of Peaks 7, 8, and 9 using Five Different Buffer Concentrations*

Buffer conc.	N_7	N_8	N_9
12.5 mM tetraborate–5 mM phosphate	73,600	24,300	48,800
12.5 mM tetraborate–12.5 mM phosphate	193,400	146,600	191,600
20 mM tetraborate–10 mM phosphate	95,200	49,800	50,300
15 mM tetraborate–15 mM phosphate	28,500	30,400	16,400
20 mM tetraborate–20 mM phosphate	43,300	31,500	45,900

* Conditions same as Figure 2.

Table II. Efficiencies of Peaks 7, 8, and 9, and Resolutions of Peaks 7 and 8 at Three Different pH Values*

	pH 8	pH 9	pH 10
N_7	30,000	103,000	193,400
N_8	50,700	71,000	146,600
N_9	38,300	80,000	177,700
$R_{s(7,8)}$	0.64	1.12	1.58

* Conditions same as Figure 3.

at a lower pH value analyte 3 eluted after analyte 4, when the pH increased the elution order of these two analytes was reversed. The increasing anionic character of analyte 3 (p*K*a = 8.32) resulted in a decrease in binding with the anionic surfactant (22). This ionic repulsion caused an earlier elution of galanthamine at pH 10. Similar situation was observed with the peak pairs 5–6 and 7–8.

Effect of SDS concentration

In an attempt to achieve baseline separation of the nine AChEIs, the influence of the SDS concentration was investigated using 15 mM, 20 mM, 30 mM, and 40 mM SDS in a buffer solution consisting of 12.5 mM phosphate and 12.5 mM borate (pH 10). Micelles in the running BGE solution constitute a stable hydrophobic phase called pseudostationary phase (23). The more the solute interacts with the hydrocarbon interior of the negatively charged micelle the longer its migration time is (24). As demonstrated in Figure 4, an addition of SDS to the running BGE resulted in a significant increase in the resolution between the peaks when compared with the electropherogram obtained using CZE (data not shown). It has been reported that an increase in the micellar concentration decreased the electroosmotic mobility, mainly due to an increase in electrolyte viscosity (25). Likewise, as the SDS concentration increases, the distribution of the analyte between the aqueous phase and the hydrocarbon phase in the interior of the micelles increases (26). Therefore, when higher SDS concentrations were used, the electrophoretic mobilities of the solutes decreased, which, in turn, increased the migration times. In addition, higher SDS concentrations resulted in a loss of efficiency and a decrease in resolution between peaks 7 and 8. At even higher than 40 mM SDS concentrations, components 7 and 8 coeluted (data is not shown). It should be mentioned that when 15 and 20 mM SDS concentrations were used, baseline separation of the nine analytes was achieved. However, the electropherogram obtained when a 15 mM SDS concentration was used demonstrates a bad peak shape when compared with the electropherogram obtained with 20 mM SDS concentration. With these results in mind, the 20 mM SDS concentration was chosen as the optimum concentration.

Effect of applied voltage

Theoretical equations for efficiency and resolution suggest the use of high electric fields (24). However, Joule heating sometimes limits the benefits of this approach. The influence of the applied voltage (10, 20, and 30 kV) on the efficiency, resolution, and analysis time of the AChEIs was evaluated using a background electrolyte of 12.5 mM phosphate, 12.5 mM borate and 20 mM SDS. The best results in regards to resolution and analysis time were obtained at 30 kV. As anticipated, at a higher applied voltage the analysis time was considerably shortened (from 62 min at 10 kV to 14 min at 30 kV) due to an increase in both the EOF and the electrophoretic mobility of the analytes. In addition, at all applied voltages, the resolution of all the peaks, and particularly the peaks 7 and 8, was above 1.5. The resolution of the latter two peaks was 1.58 when 30 kV were applied, while it increased to 2.26 at an applied voltage of 10 kV. Moreover, at 30 kV, no Joule heating effects that would have limited the analysis

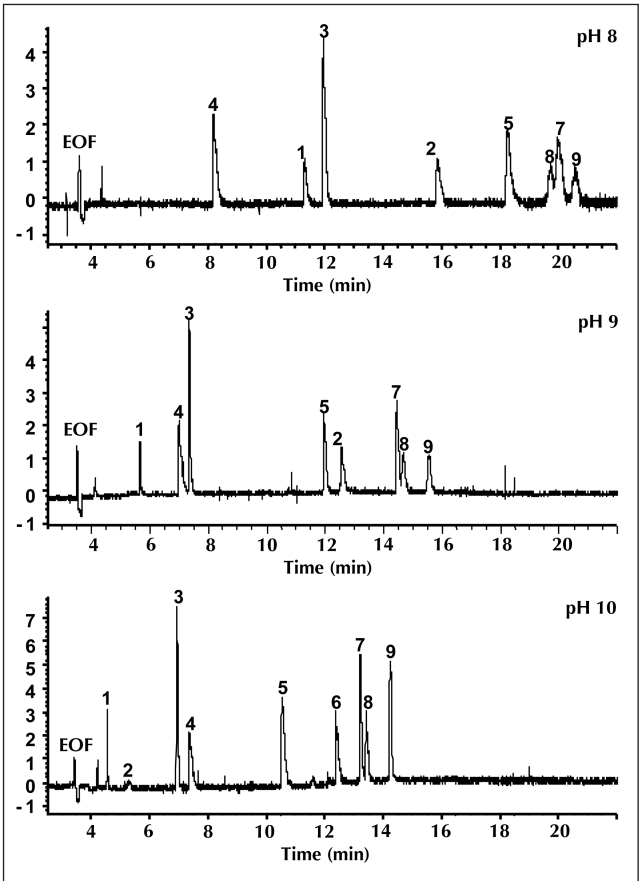

Figure 3. Effect of pH value on the separation of the nine AChEIs. Conditions: same as Figure 2B, except pH value was varied from 8 to 10. The peaks identification is shown in Figure 1.

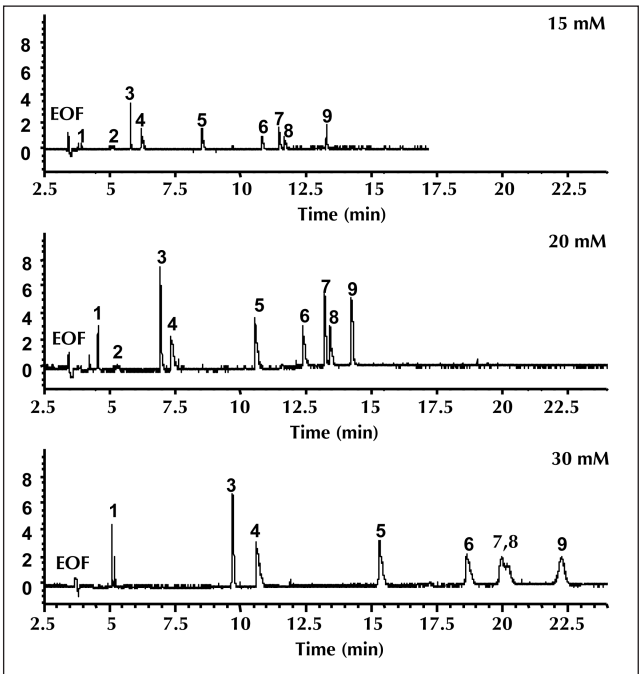

Figure 4. Effect of SDS concentration on the separation of the nine AChEIs. Conditions: same as Figure 2B, except SDS concentration was varied from 15 to 30 mM.

were observed. The resulting electrophoretic currents, due to the applied voltages 30, 20, and 10 kV were 60, 35, and 17 µA, respectively. Based on these results, the optimal voltage was set at 30 kV. The latter applied voltage ensured baseline separation, shorter migration times and acceptable current generation.

Effect of temperature

In MEKC, temperature should be strictly controlled in order to obtain reproducible results. This is due to migration time's dependence on temperature (20). Temperature control can also be used as a parameter for optimizing separation conditions. An increase in temperature from 10°C to 35°C was investigated for the separation of AChEIs. As anticipated, the retention time decreased with an increase in temperature. This increase in temperature resulted in a reduced analysis time (from 26 min at 10°C to 11 min at 35°C) due to an increase in the EOF that is probably caused by a decrease in electrolyte viscosity. In addition, since the time window became narrower, resolution deteriorated at a temperature higher than 25°C, particularly for the peak pair 7 and 8. The resolution of these peaks decreased from 1.58 to 0.92 at 25°C and 35°C, respectively. Therefore, the capillary temperature was maintained at 25°C since at this temperature all nine AChEIs were successfully resolved.

Effect of injection time

In this study, injection time was a limiting factor. At a constant injection pressure of 30 mbar, peak-splitting phenomenon was observed when the injection time was 3 s or longer. Peak splitting is a common phenomenon in MEKC (27,28). In the literature, several possible causes of peak splitting are mentioned, such as injection time, SDS and analyte concentration and hydrophobicity of the analytes (27). In our experiments, the injection time was likely the only reason that caused peak splitting. Rafols et al. proposed a hypothesis in order to explain this phenomenon (28): when the sample plug is drawn into the capillary, the analytes at both ends of the injection plug are surrounded by high concentrations of SDS micelles. Immediately, analytes begin partitioning into the micelles and two regions of high analyte concentration are developed. If injection time is short, these two regions will mingle because of radial diffusion. However, when injection time is long enough, the distance between the two high-analyte-concentration zones is still obvious, even when radial diffusion takes place. The latter causes the formation of double peaks. With this hypothesis in mind and based on the results that were obtained, 1 s was chosen as the optimum injection time in order to avoid the splitting of the analyte peaks.

MEKC optimum separation

Figure 5 demonstrates the baseline separation of the nine AChEIs. At this point it is important to mention that despite of the various method parameters that were examined in this study, the intensity of the peak that corresponds to the AChEI eseroline is too low. In literature, it is stated that physostigmine is unstable, and it is hydrolyzed enzymatically or in an aqueous medium at a high pH in order to produce eseroline (9,11). Subsequently, oxidation of eseroline yields the compound rubreserine. Therefore, the small intensity of the peak is likely due to the oxidation of eseroline. Rubreserine was not detected under these experimental conditions. The peak of physostigmine was not affected due to the presence of pyridostigmine bromide and neostigmine bromide in the mixture of the analytes. Studies have shown that the addition of these compounds prevents the decomposition of physostigmine (33).

Validation

The MEKC method was validated by using the terms of limit of detection (LOD), limit of quantitation (LOQ), and precision. LOD and LOQ were determined as three and ten times, respectively, the standard deviation of the blank signal related to the sensitivity, which was expressed by peak area to concentration. The standard deviation was obtained from five consecutive measurements of the area of a peak that corresponds to rivastigmine sample at a concentration of 6.5 µg/mL. The LOD and LOQ were 0.5 µg/mL and 1.7 µg/mL, respectively.

Precision is an important factor for the evaluation of a method. Precision was evaluated by using the relative standard deviation (RSD) values of the migration times and the areas of the nine-analyte peaks (34,35). The run-to-run RSD values were obtained from five consecutive electrophoresis runs. Most of the RSD values of the migration times were below 2%, indicating very good run-to-run reproducibilities. In the case of peaks 2 and 4, the RSD values were even below 1%. The RSD values of the areas ranged from 2.7% to 7.7%. In addition, the RSD values of the migration time and the area of the peak that corresponds to rivastigmine added in the blood sample were 0.7% and 2.1%, respectively. These values, which were obtained from five replicate analyses, demonstrate an excellent reproducibility.

Application

The proposed method was used for the determination of rivastigmine in a real blood sample. Optimum conditions were applied to a whole blood sample that was obtained from an AD patient who was not under medication. The sample was spiked with rivastigmine in order to establish the ability of the method

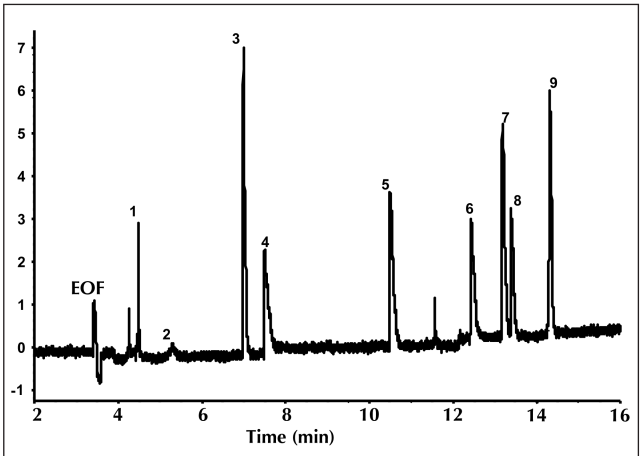

Figure 5. Optimized electropherogram for the separation of the nine AChEIs. Conditions: 12.5 mM phosphate and 12.5 mM tetraborate (pH 10); 20 mM SDS; pressure injection, 30 mbar for 1 s; applied voltage, 30 kV; temperature, 25°C; fused-silica capillary, 64 cm (55.5 cm effective length) × 50 µm i.d.; detection, 214 nm. The peaks identification is shown in Figure 1.

to separate the drug compound from all the others components. If blood sample is directly injected into the capillary, some components that exist in the sample can be absorbed to the capillary wall and deteriorate the performance of the column (13). Thus, the blood sample was initially diluted ten folds with the buffer solution [12.5 mM Na_2HPO_4/12.5 mM $Na_2B_4O_7$/20 mM SDS (pH 10)] (Figure 6A). Then, the sample was spiked with 25 µg/mL of rivastigmine (Figure 6B). As illustrated in Figure 6B, rivastigmine was separated from the sample matrix within 13.4 min. The presence of rivastigmine was also confirmed by further spiking, which resulted in an increase in peak intensity (Figure 6C). It should also be mentioned that when rivastigmine was in the blood sample, it eluted at the same time it eluted when it was in the mixture of the nine standard AChEIs. Therefore, the ability of the MEKC method to separate rivastigmine from other blood components was established.

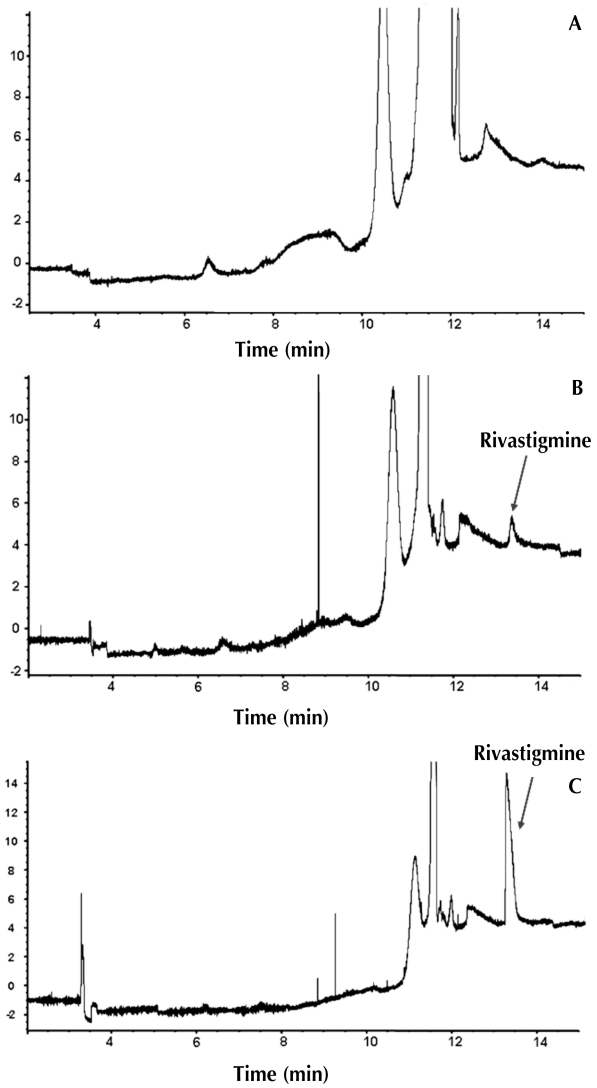

Figure 6. Determination of rivastigmine in a real blood sample under optimum conditions. (A) Blank blood sample, (B) blood sample spiked with 25 µg/mL of rivastigmine, (C) further spiking of rivastigmine in the blood sample. Ten-fold dilution of blood sample with the buffer solution. Conditions: same as Figure 5.

Conclusion

This study has fully demonstrated the capability of a MEKC method to efficiently separate nine AChEIs. In the process of method development, the main parameters that needed to be optimized were the concentrations of the BGE and the surfactant, the pH, the applied voltage and the temperature. The use of an organic modifier proved to be unnecessary, since it resulted in poor resolution and long migration times. Baseline separation of the nine AChEIs was achieved in less than 15 min by using 12.5 mM Na_2HPO_4, 12.5 mM $Na_2B_4O_7$, and 20 mM SDS at pH 10, an applied voltage of 30 kV and a temperature of 25°C. In addition, the peak shapes were considerably improved when the injection size was short (30 mbar for 1 s). The reproducibility of the method also proved to be very good with RSD values of the migration times and the peak areas of less than 2% and 6%, respectively, in most cases. Successful determination of rivastigmine was also achieved when the MEKC method that was described in this study was applied to a spiked blood sample. Considering the results mentioned above, MEKC proved to be a fast and an applicable method for the separation of AchEIs.

Acknowledgements

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