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Article

Separation of Acetylcholinesterase Inhibitors Using Polymeric Surfactants in Polyelectrolyte Multilayer Coatings

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The main objective of this study is the use of polymeric surfactants in polyelectrolyte multilayer (PEM) coatings for the separation of the pharmaceutical substances acetylcholinesterase inhibitors (AChEIs). AChEIs are used for the treatment of Alzheimer's Disease and Myasthenia Gravis. In the open-tubular capillary electrochromatography (OT-CEC) mode, the PEM coating is evaluated using nine AChEIs. Optimal conditions are established by altering several experimental parameters such as the pH of the background electrolyte (BGE), the anionic polymer for the PEM coating, the concentration of NaCl, which is used as an additive in the polymer deposition solutions, the number of bilayers, the deposition time, and the concentration of the polymeric surfactant. 25 mM $NaH_2PO_4.H_2O$ and 25 mM Na_2HPO_4 at pH 7 is used as BGE. Two bilayers of poly(diallyl dimethyl ammonium chloride) and poly(sodium *N*-undecanoyl L-leucinate) provide a baseline separation of all nine analytes in less than 4.5 min. Run-to-run reproducibility studies are also performed, and the relative standard deviation values of the migration times of the nine-analyte peaks are less than 2%. In addition, day-to-day, week-to-week and capillary-to-capillary reproducibilities are evaluated, and the relative standard deviation values of the electroosmotic flow are less than 2%. Finally, using the PEM coating approach, we were able to perform more than 150 runs in the same column. Neither the addition of the polymeric surfactant to the mobile phase, nor the reconstruction of the coating was necessary.

Introduction

Over the last decade, the use of polymeric surfactants in chiral and achiral electrophoretic separations has attracted considerable attention. Polymeric surfactants can be used in a polyelectrolyte multilayer (PEM) coating procedure in the open-tubular capillary electrochromatography (OT-CEC) mode (1–7), and in a background electrolyte (BGE), as a stable pseudostationary phase in the micellar electrokinetic chromatography (MEKC) mode for achiral (7–9) and chiral separations (7, 10–16).

In OT-CEC, the stationary phase is immobilized on the inner surface of the capillary (17). One of the most recently used approaches for modifying the capillary wall is the PEM coating. The coating is constructed in situ by alternating rinses of positively and negatively charged polymers, where the anionic polymer can be a polymeric surfactant. PEM coatings are physically adsorbed to the capillary wall via an ion exchange process. The coating procedure is simple, and it can provide an excellent reproducibility, a great stability and a long lifetime (1, 3, 5, 6). Another important advantage of the PEM coating is that it makes the capillary electrophoresis (CE) system more amenable to coupling with mass spectrometry (MS). This is because the polymers are adsorbed onto the capillary wall, and therefore, there is very little detection interference of the polymers with the analytes of interest (2, 18). Finally, since the polymeric surfactant is coated onto the capillary, there is less consumption of the reagent, when compared with MEKC, where the polymeric surfactant is used as an additive in the BGE. One of the most commonly used cationic polyelectrolytes, which was also used in this study for the construction of the PEM coating is the poly(diallyl dimethyl ammonium chloride) (PDADMAC) (1, 5, 6, 17, 18). For the construction of the negatively charged layer, different polymeric surfactants (monopeptides and dipeptides) have been examined.

Kapnissi et al. (5) examined the performance of the PEM coating by use of seven benzodiazepines, which were almost resolved in less than 21 min. In their studies, poly (sodium *N*-undecanoyl-L-glycinate), poly(L-SUG), was used as the anionic polymer and PDADMAC was used as the cationic polymer. The multilayer coatings used in this study consisted of ten layer pairs. The run-to-run, day-to-day, week-to-week, and capillary-to-capillary reproducibilities of the EOF were excellent with RSD values of less than 1% in all cases. In addition, the PEM coated capillaries exhibited high stability against solutions with extreme pH values.

Kamande et al. (6) used a PEM coating for the separation of phenols and benzodiazepines. For the construction of a single bilayer PEM coating they used the polymeric surfactant, poly(sodium undecylenic sulfate), poly(SUS), and PDADMAC. Benzodiazepines and phenols were completely resolved in 26 and 18 min, respectively.

In this report, we describe the use of polymeric surfactants in PEM coatings for the separation of nine acetylcholinesterase inhibitors (AChEIs). AChEIs are used medically for the treatment of Alzheimer's disease, one of the most common causes of mental deterioration in elderly people (19), as well for myasthenia gravis, an autoimmune disease characterized by fatigable muscle weakness (20).

The separation of AChEIs has been of great importance in many industries, particularly the pharmaceutical industry. Different analytical methods have been reported for the determination of different AChEIs in biological samples. These methods include high-performance liquid chromatography (HPLC) (21, 22), gas chromatography-mass spectrometry (GC–MS) (23, 24) and CE (25, 26). Recently, Kapnissi et al. (27) reported the simultaneous separation of the nine AChEIs by use of MEKC. The separation was achieved in less than 15 min. However, in our study, the PEM coating was able to achieve a baseline separation with highly efficient peaks in less

than 4.5 min. In this report, the influence of several parameters that are used to obtain a more efficient and reproducible separation of the nine AChEIs is examined.

Experimental

Apparatus and conditions

All experiments were carried out on an Agilent, G1600A Capillary Electrophoresis System, which consisted of a regulated high-voltage power supply and a diode-array detector (DAD) for UV detection. The DAD was set at 214 nm for the detection of the nine AChEIs. The instrument was also controlled by Hewlett-Packard CE Chemstation software for the collection and integration of all experimental data. Fused-silica capillaries of 64.0 cm (55.5 cm effective length) Ÿ 50 μm i.d. and 360 μm o.d. were purchased from Polymicro Technologies (Phoenix, AZ). The temperature of the capillary cassette ranged from 10°C to 25°C, and the applied voltage ranged from 10 to 30 kV. The samples were injected by pressure at 30 mbar for 1 s to 7 s.

Reagents and chemicals

Pyridostigmine bromide, edrophonium chloride, neostigmine bromide, eseroline, physostigmine, galanthamine hydrobromide, *N*-methyl-physostigmine, 1,5-Bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide, sodium acetate (CH_3COONa), PDADMAC ($M_r = 200\ 000–350\ 000$) and poly (sodium 4-styrenesulfonate) (PSS) ($M_r = 6 \times 10^6$) were purchased from Sigma-Aldrich. Rivastigmine was extracted from Exelon capsules according to a procedure previously described by Kavalirova et al. (28). Exelon capsules (Novartis Pharmaceuticals, Basel, Switzerland) were kindly donated by the Cyprus Institute of Neurology and Genetics. The structures of the analytes used in this study are shown in Figure 1. Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4.H_2O$ and sodium phosphate dibasic, Na_2HPO_4 were purchased from Fischer Scientific. Polymeric surfactants, poly(L-SUL), poly(sodium *N*-undecanoyl L-leucinate); poly(L-SUV), poly(sodium *N*-undecanoyl L-valinate) and poly(LL-SUVG), poly(sodium *N*-undecanoyl-LL-valyl-glycinate) were synthesized at the Louisiana State University by I. M. Warner's research group (5).

Sample and buffer preparation

Analytical standard stock solutions of the nine analytes were prepared in H_2O–MeOH (1:1) at concentrations of ~0.9 mg/mL each in order to give a final concentration of 0.1 mg/mL in the mixture. The mobile phase consisted of $NaH_2PO_4.H_2O$ and/or Na_2HPO_4 depending on the pH. The BGE was prepared by dissolving the appropriate amount of the sodium phosphate in 25 mL of deionized water and the pH was adjusted by use of 1 M sodium hydroxide (NaOH).

Procedure for PEM coating

First, a detection window of 0.5 cm was prepared by burning off the external polyimide capillary coating. PEM coating was constructed by deposition of the polymer solutions on the

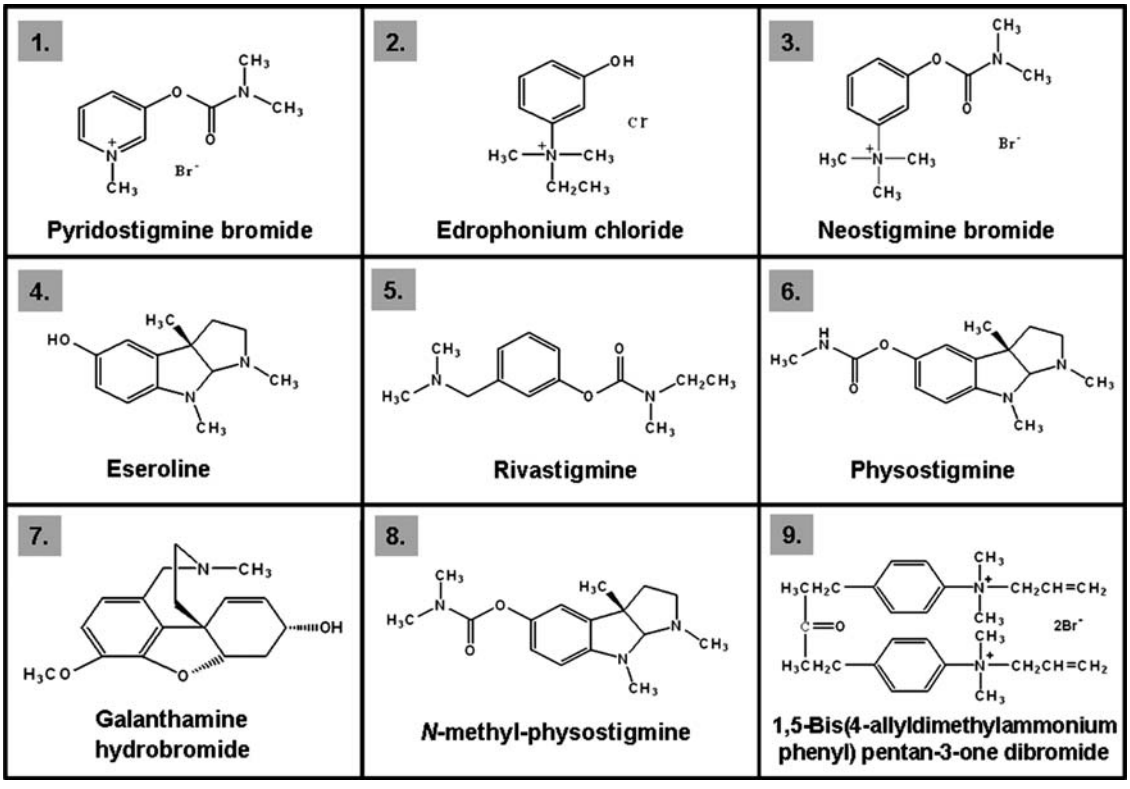

Figure 1. Structures of the AChEIs.

inner capillary wall. Each polymer solution contained 0.5 % (w/v) polymer. The fused-silica capillary was initially conditioned with water for 5 min, then with 1 M NaOH for 60 min, and again with water for 15 min. Next, the first layer of the cationic polymer was constructed by rinsing the PDADMAC polymer solution through the capillary for 5 min followed by a water rinse for 5 min. The anionic polymer solution was then flushed for 5 min followed by a 5-min rinse with water. A layer of cationic polymer plus a layer of anionic polymer constitute a bilayer. After the construction of the coating, the capillary was flushed with the BGE for 30 min until a stable baseline and a stable current were obtained. The columns were conditioned with the BGE for 5 min between injections.

Results and Discussion

In this study, several experimental parameters were investigated, including the type of the anionic polymer, the number of bilayers, the deposition time of the polymer solutions, the concentration of the polymeric surfactants, and the pH of the BGE.

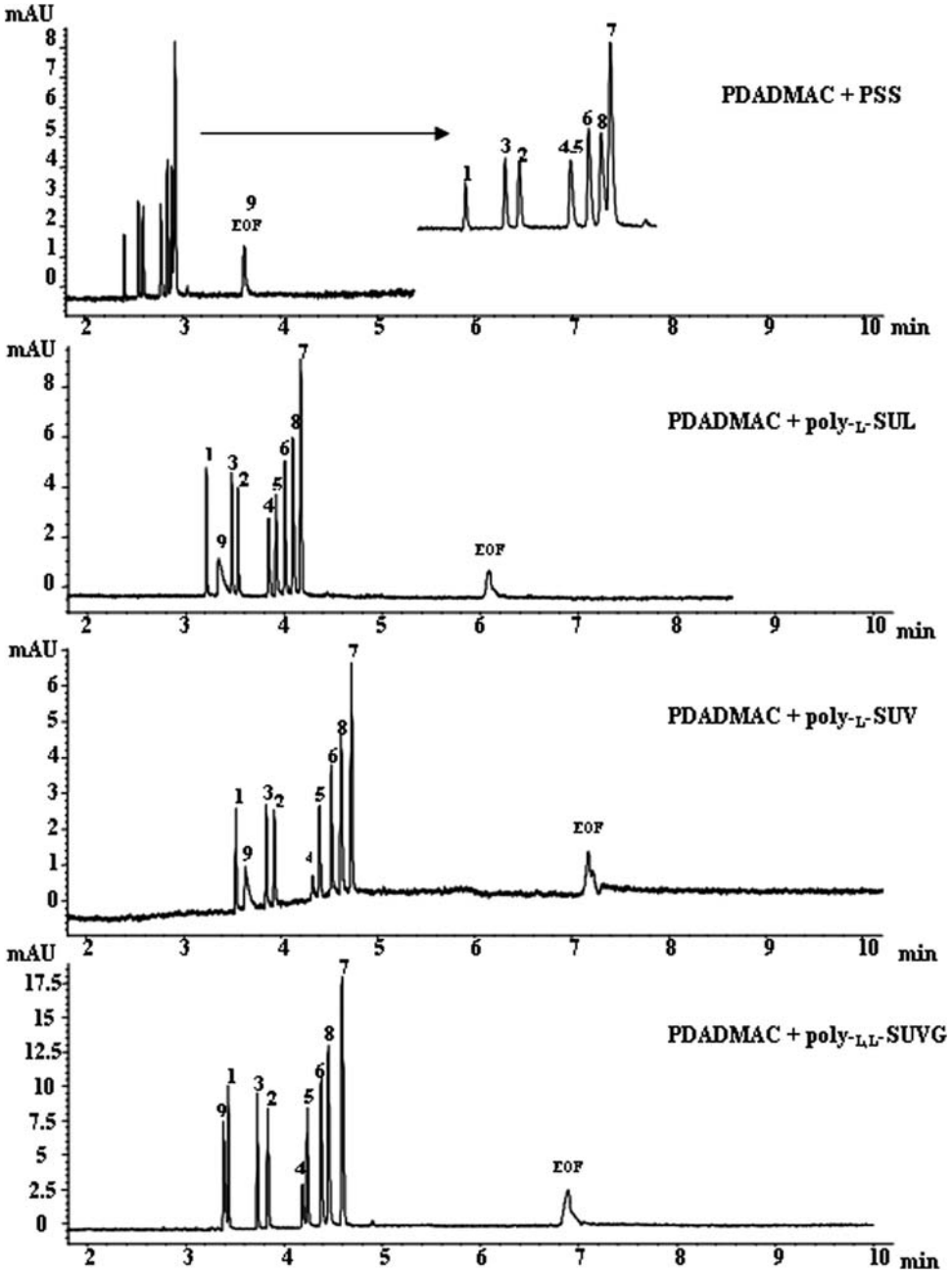

Figure 2. Effect of anionic polymers on the OT-CEC separation of AChEIs. Conditions: BGE, 25 mM monobasic/ 25 mM dibasic sodium phosphate (pH 7); fused silica capillary, 64.0 cm (effective length 55.5 cm ¥ 50 μm i.d.); detection, 214 nm; applied voltage, 30 kV; temperature, 25°C; pressure injection, 30 mbar for 3 sec; 2 bilayer, 0.5 % (w/v) PDADMAC and 0.5 % (w/v) anionic polymer.

Effect of anionic polymer

A comparative study was initially performed in order to investigate the separation of AChEIs by the use of PSS, or polymeric surfactants as the anionic layers in the PEM coating. As demonstrated in Figure 2, the three polymeric surfactants allowed better discrimination of all nine analytes than the PSS. The use of the monopeptides poly-L-SUL and poly-L-SUV resulted in a better separation of the AChEIs. The analysis time though was increased when the poly-L-SUV was used. The analytes 1 and 9 were not completely resolved, when the dipeptide poly-L,L-SUVG was used as the anionic polymer (resolution factor of 1.40). Thus, taking into account the resolution (Rs) of the analyte pairs 4–5 (1.84) and 1–9 (3.02), and the analysis time, the poly-L-SUL was considered as the optimum polymeric surfactant in the PEM coating.

Effect of polymeric surfactant concentration

Another parameter that was investigated was the concentration of the polymeric surfactant poly-L-SUL. Two different concentrations were examined, 0.5% (w/v) and 0.75% (w/v), while the other parameters remained constant. At a concentration of 0.75% (w/v) poly-L-SUL, even though the elution order remained the same, the analytes 4 and 5 co-eluted (Figure 3). An increase in analysis time and a decrease in electroosmotic mobility were also observed. Therefore, the concentration of 0.5% (w/v) was the optimum, since it resulted in shorter analysis time and better resolution (Rs of peaks 1 & 9: from 1.38 to 3.02; Rs of peaks 4 & 5: from 0 to 1.84).

Effect of NaCl concentration

It has been shown that the addition of NaCl in the polymer deposition solutions results in an increase in the thickness of the coating (29, 30). Some studies have also demonstrated an increase in resolution (1, 18). For this study, the polymer deposition solutions consisted of 0.5% (w/v) polymer and different concentrations of NaCl. It was observed that the addition of 0.01 M, 0.05 M, and 0.10 M NaCl did not increase the resolution of the peaks, and the difference in analysis time was not significant (data not shown). At a higher concentration of NaCl, the migration times of the analytes increased, while the resolution decreased, since some of the analytes co-eluted. As far as the efficiency is concerned, when no NaCl was used, the efficiency was above 8,000, whereas the addition of NaCl decreased this parameter significantly, and, in most cases, it dropped it even below 4,000. In addition, when 1.0 M NaCl

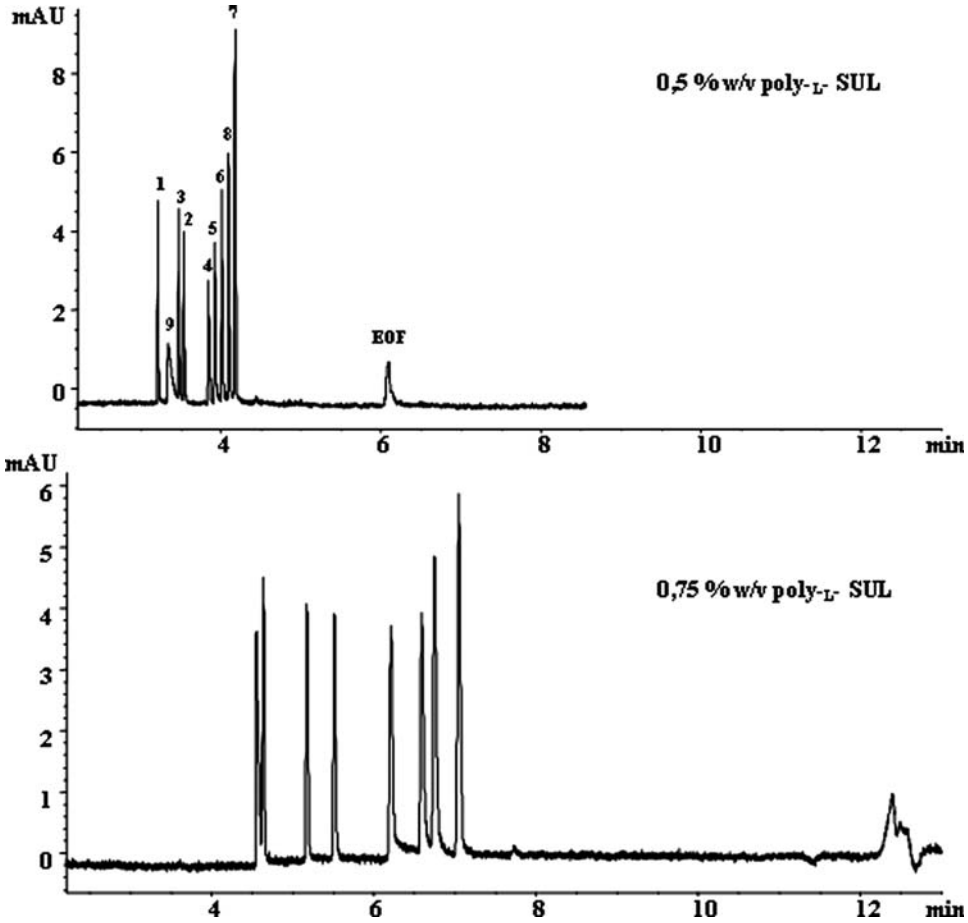

Figure 3. Effect of polymeric surfactant concentration on the OT-CEC separation of AChEIs. Conditions: same as Figure 2, except polymer (poly-L-SUL) concentration was varied.

was added to the polymer deposition solutions, it was very difficult to maintain the stability of the current, probably due to an increase in the thickness of the coating that can clog the capillary or due to the Joule heating effect. Based on these results, the addition of NaCl in the polymer deposition solutions proved to be unnecessary.

Effect of bilayer number

The effect of the number of bilayers was examined in regard to resolution and analysis time. It has been shown that an increase in the bilayer number results in an enhanced film thickness and retention time (1, 29, 30). Two, 6, 8 and 10 bilayers were constructed, and the poly-L-SUL was used as the anionic polymer at a concentration of 0.5 % (w/v). As illustrated in Figure 4, both the efficiency and the resolution of the peaks decreased, while the analysis time increased when more bilayers were constructed. In particular, the resolution of the peaks 4–5 decreased dramatically when 8 bilayers were used (from 1.84 to almost 0), and the R_s of the peaks 1 and 9 decreased significantly when 10 bilayers were constructed (from 3.02 to 0.66). Based on these results, and based on the

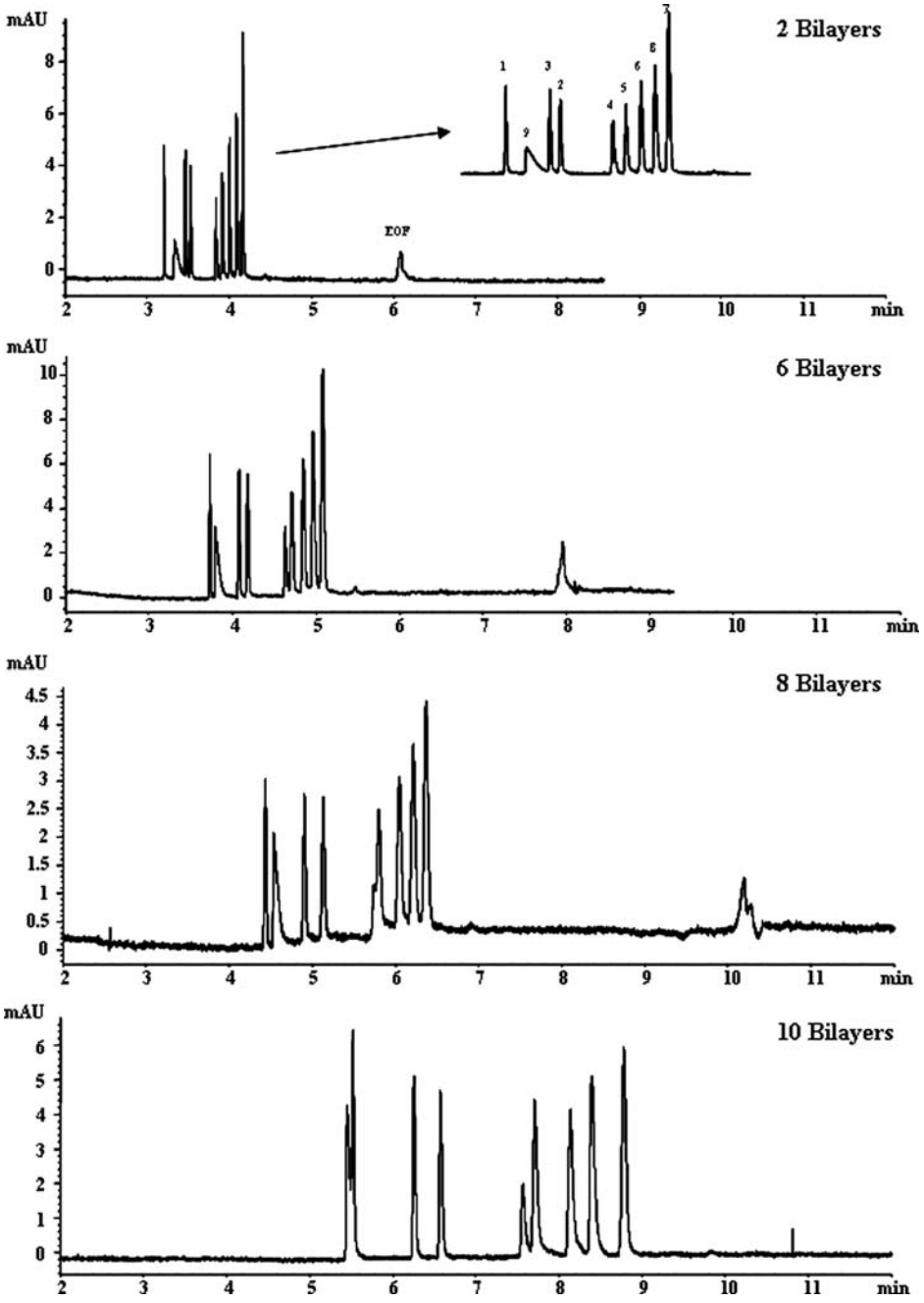

Figure 4. Effect of bilayer number on the OT-CEC separation of AChEIs. Conditions: same as Figure 2, except bilayer number was varied.

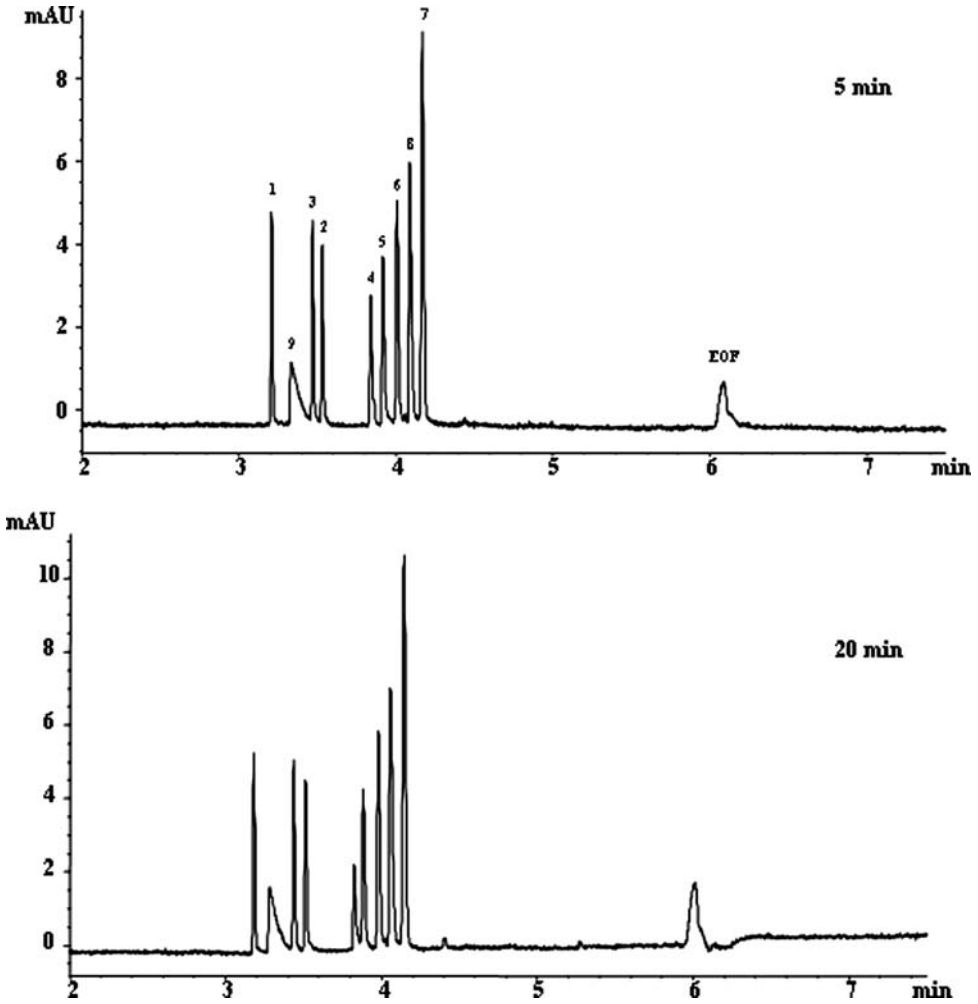

Figure 5. Effect of polymer deposition time on the OT-CEC separation of AChEIs. Conditions: same as Figure 2, except polymer deposition time was varied.

fact that the construction of 6, 8 and 10 bilayers is time-consuming, 2 bilayers proved to be the optimum for the separation of the AChEIs.

Effect of polymer deposition time

For this study, the four layers of the cationic and the anionic polymers were deposited by flushing the PDADMAC and the poly-L-SUL solutions for 5 min and 20 min. After each polymer deposition, a 5-min water rinse was performed. Five minute water and 20-min depositions did not demonstrate a change in resolution and selectivity (Figure 5). In addition, the analysis time and the intensities of the peaks remained almost the same, even after flushing the polymer deposition solutions for 20 min.

Effect of pH

The pH of a BGE plays an important role in the separation of ionic analytes. The influence of the pH on the resolution and analysis time of the AChEIs was examined by using three different BGEs. For the pH values of 5, 7 and 10, a BGE of 50 mM $NaH_2PO_4.H_2O$, a BGE of $NaH_2PO_4.H_2O$–Na_2HPO_4, and a BGE of Na_2HPO_4 were used, respectively. At pH 10, the resolution decreased, and six out of the nine analytes coeluted (Figure 6). At pH 5, no more than three peaks eluted even at 40 min. This suggests that at low pH values the analytes of interest are retained by the coating. Based on these observations, the pH 7 was chosen as the optimum pH.

Reproducibilities

Reproducibility is an important factor for the evaluation of the coating performance. The run-to-run reproducibility was evaluated by calculating the relative standard deviation (RSD) values of the migration times of the nine-analyte peaks. The RSD values were obtained from ten consecutive electrophoresis runs, and they ranged from 0.58% to 1.86%. The day-to-day, week-to-week, and capillary-to-capillary RSD values of the electroosmotic flow were obtained from four replicate analyses, three replicate analyses, and sixteen runs (four consecutive runs performed in four capillaries), respectively. In this case, the RSD values ranged from 1.82% to 1.98%.

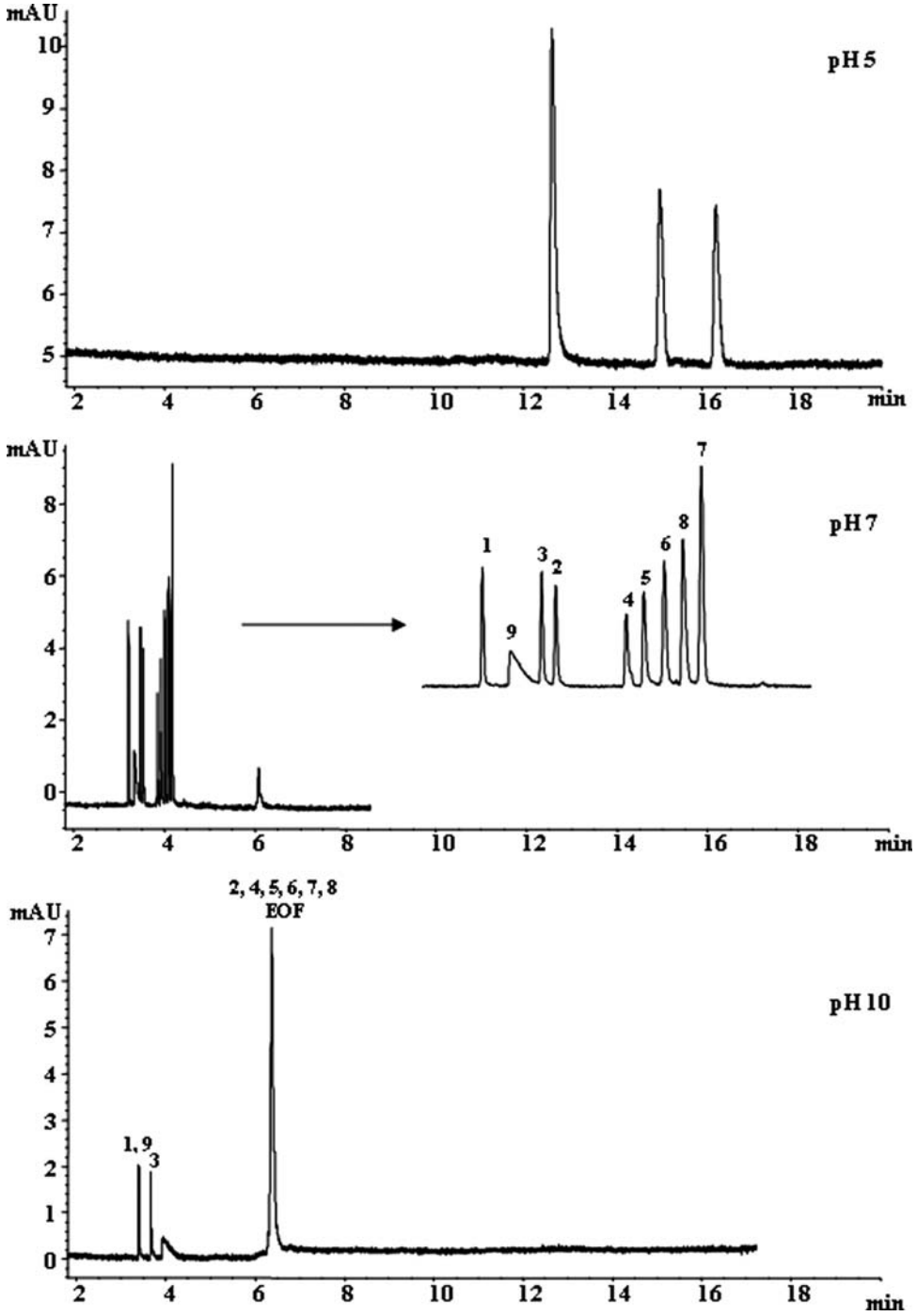

Figure 6. Effect of pH on the OT-CEC separation of AChEIs. Conditions: same as Figure 2, except pH and BGEs were varied. (a) 50 mM $NaH_2PO_4.H_2O$ at pH 5, (b) 25 mM $NaH_2PO_4.H_2O$ and 25 mM Na_2HPO_4 at pH 7, and (c) 50 mM Na_2HPO_4 at pH 10.

Application

The optimum OT-CEC method was applied to a blood sample that was obtained from an AD patient who was not under medication. The blood sample was first diluted ten folds with the BGE solution (25 mM $NaH_2PO_4.H_2O$ and 25 mM Na_2HPO_4 at pH 7), and then, it was injected into the CE system (Figure 7A). The blood sample was then spiked with 25 μg/mL of rivastigmine. Figure 7B demonstrates the separation of rivastigmine from the sample matrix. The presence of rivastigmine was confirmed by further spiking, which, in turn, increased the peak intensity. Therefore, the optimum OT-CEC method proved to be capable of separating the drug compound from other components that might exist in the blood sample.

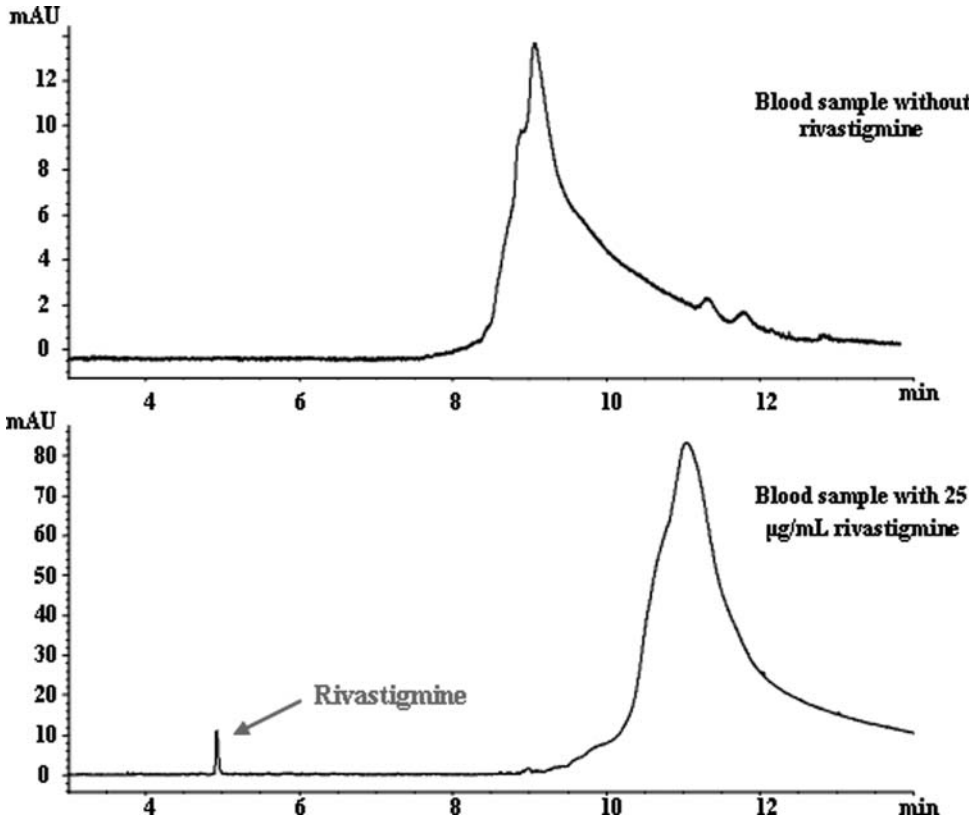

Figure 7. Determination of rivastigmine in a blood sample using the optimum separation conditions. (a) Blank blood sample, (b) Blood sample spiked with 25 μg/mL of rivastigmine. Conditions: same as Figure 2.

Conclusion

In this study, we were able to employ polymeric surfactants in PEM coatings for the separation of nine AChEIs. The polymeric surfactant poly-L-SUL proved to be the best discriminator for the separation of the analytes under study. A baseline separation with highly efficient peaks was achieved in less than 4.5 min by using 25 mM $NaH_2PO_4.H_2O$ and 25 mM Na_2HPO_4 at pH 7, and a PEM coating that consisted of two bilayers of PDADMAC and poly-L-SUL. The use of NaCl in the polymer deposition solutions proved to be unnecessary, since it resulted in poor resolution and efficiency, long migration times, and unstable current. In addition, the reproducibility of the coating was very good with RSD values of less than 2%.

Acknowledgments

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