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Application of carboxymethyl-β-cyclodextrin as a chiral selector in capillary electrophoresis for enantiomer separation of selected neurotransmitters

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Abstract

The aim of this work was to optimize conditions for capillary electrophoresis separation of different neurotransmitters (serotonin, phenylalanine, dopamine, adrenaline, ephedrine, propranolol and DOPA) in a single run, including separation of existing enantiomers. As chiral selectors added to the borate background, electrolyte unsubstituted α-, β- and γ-cyclodextrins (CDs), methyl-, dimethyl-, and trimethyl-substituted β-CDs, and hydroxypropyl-substituted α-, β- and γ-CDs were examined. Also carboxymethyl-β-CD and succinyl-β-CD were used for this purpose. In addition to the kind and concentration of chiral selector, some other experimental factors also have been optimized, such as concentration of borate buffer, content of methanol, pH of electrolyte, method of sample introduction into the capillary and washing procedure between consecutive runs. The best results were obtained using 20 m*M* carboxymethyl-β-CD in borate buffer of pH 7.5 as running electrolyte and hydrostatic injection. The obtained sensitivity of response (peak height) varied from 0.4 for adrenalines to 2.3 mAU mM^{-1} for propranolols. The concentration detection limits ($S/N=3$) were in the range from 0.04 m*M* for propranolols to 0.2 m*M* for adrenalines. The resolution obtained in optimized conditions in a single run was from 0.75 for adrenalins and 1.0 for propranolols up to 2.0 for ephedrines. The developed method was employed for determination of these analytes in brain tissue extracts.

Keywords: Enantiomer separation; Background electrolyte composition; Pharmaceutical analysis; Cyclodextrins; Neurotransmitters

1. Introduction

Determination of neurotransmitters and their enantiomers is of great importance for biomedical research, medical diagnostics and the pharmaceutical industry. These compounds play a very important role in control and regulation of a variety of functions in both the central and peripheral nervous

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system. They are also known to have endocrine and exocrine influence on the production of hormones [1]. Catecholamines also belong to this group of compounds. Qualitative and quantitative analysis of catecholamines plays an important role in the diagnosis and treatment of diseases such as, for instance, Parkinsons' disease. Concentration of catecholamines in blood plasma varies between 1 and 800 pg ml^{-1}, with typical levels in cerebrospinal fluid of 0–300 μg ml^{-1}, in urine of 100–400 μg ml^{-1} and in brain tissue of 10–1000 μg g^{-1} [1].

Various capillary electrophoretic methods have been employed, so far, for separation of neurotransmitters, such as capillary zone electrophoresis, micellar electrokinetic capillary chromatography and capillary electrochromatography. They were used with various detection methods including amperometric, laser-induced fluorescence, and luminescence detection. In capillary electrophoresis separation of neurotransmitters, the most commonly used electrolytes, has been based on borate, phosphate and citrate, but mixed buffers such as phosphate–borate, Tris–phosphate and acetate–borate have also been reported. Additives for modification of migration time are also important, with methanol, acetonitrile, urea and crown ethers most often used. The most widely used chiral selectors in the separation of neurotransmitters have been unsubstituted β-, and γ-cyclodextrins [2–4] and substituted β-cyclodextrins (CDs): methyl-β-CD [5], dimethyl-β-CD [3,6–10], trimethyl-β-CD [5], carboxymethyl-β-CD [11–15], hydroxypropyl-β-CD [3,16,17], sulfobutyl-β-CD ether [6,18,19], hydroxyethyl-β-CD [20] and polymer β-CD [21].

So far, various works have reported separations of up to three different catecholamines and neurotransmitters in a single run. The aim of this study is to present the effect of different kinds of cyclodextrins, both neutral and substituted, and to optimize CE conditions for simultaneous chiral separation of several catecholamines and other selected neurotransmitters. The aim is to perform the analysis of complex mixtures in a single run, including separation of existing enantiomers.

Data for serotonine (SER), dopamine (DP), adrenaline (AD), ephedrine (EPH), propranolol (PRO), phenylalanine (PHE) and dihydroxyphenyloalanine (DOPA) and their enantiomers are presented in this paper. In addition the experimental conditions have been optimized. The choice of chiral selector and the control of the background electrolyte have been particularly addressed. Other experimental factors such as sample introduction into the capillary, applied voltage and procedure of capillary washing between consecutive runs have also been examined. The latter factor is of special importance for applications to real biomedical samples.

The preliminary determination of neurotransmitters in drugs and biological samples such as urine and cerebrospinal fluid demonstrates the success of the procedures described.

2. Experimental

2.1. Apparatus

Experiments were performed with two different instruments: (i) Waters Quanta 4000 with UV detection at 210 nm (Millipore, Waters Division, Milford, MA. USA); and (ii) 270A-HT CE System from Applied Biosystems (San Jose, CA, USA) with Waters System Interface Module detector (Millipore, Waters Division). Separations were carried out with fused-silica capillaries from Chromatography-Service (Langerwehe, Germany) and Polymicro Technologies (Phoenix, AZ, USA). Both types were 75 cm (50 cm effective length)×50 μm I.D.

2.2. Capillary conditioning

Rinsing after each run for 4 min with background electrolyte (BGE) was not sufficient, and large positive errors were obtained. A washing cycle including HCl, water, NaOH, water and BGE (each 4 min) gave some improvement, but the best reproducibility of results was obtained with the following procedure. At the start of each working day the capillary was treated with 0.5 *M* NaOH for 15 min, then with Milli-Q water, followed by the carrier electrolyte. Before each electrophoretic run the capillary was then rinsed with 0.1 *M* NaOH, then water, and then background electrolyte for 4 min. At the end of experiment the capillary was rinsed for 15 min with Milli-Q water.

2.3. Chemicals

Non-substituted α-, β-, and γ-cyclodextrins were purchased from Waclier-Chemie (Munich, Germany). Methyl-β-CD (M-β-CD), dimethyl-β-CD (DM-β-CD), trimethyl-β-CD (TM-β-CD), carboxymethyl-β-CD (CM-β-CD), hydroxypropyl α-, β-, and γ-CDs (HP-α, HP-β, HP-γ-CD) and succinyl-β-CD (Succ-β-CD) were purchased from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Sodium tetraborate and methanol were purchased from Merck. Concentrated sodium tetraborate was prepared in Milli-Q water (Millipore, Bedford, MA, USA) every week. Solutions of analytes and background electrolytes were prepared each day.

3. Results and discussion

3.1. Concentration of background electrolyte

The separation of a complex mixture of enantiomers of neurotransmitters requires careful optimization of background electrolyte composition. Following previous works [5,8], a borate buffer at pH 9.2 was used as background electrolyte. The effect of borate concentration has been examined for selected catecholamines in the range of 10–50 m*M* (Fig. 1). An increase in the borate concentration decreased the EOF, which resulted in an increase in the migration time of analytes. The order of migration of analytes was a consequence of their charge and structures. Taking into consideration the protonation constants of analytes (Table 1), serotonine at pH 9.2 was almost fully protonated (cationic form), dopamine was a neutral analyte, while phenylalanine was deprotonated, and had a negative charge.

The changes in buffer concentration also affected the resolution of analytes. Increasing the buffer concentration up to 20 m*M* resulted in improvement in the separation. A further increase in buffer concentration up to 30 m*M* resulted in evident loss of resolution for dopamine and phenylalanine. Further increase in buffer concentration affected the separation and at the same time gave a peak shape distortion. It was concluded that the best buffer concentration as far as separation and analysis time were concerned, was 20 m*M*.

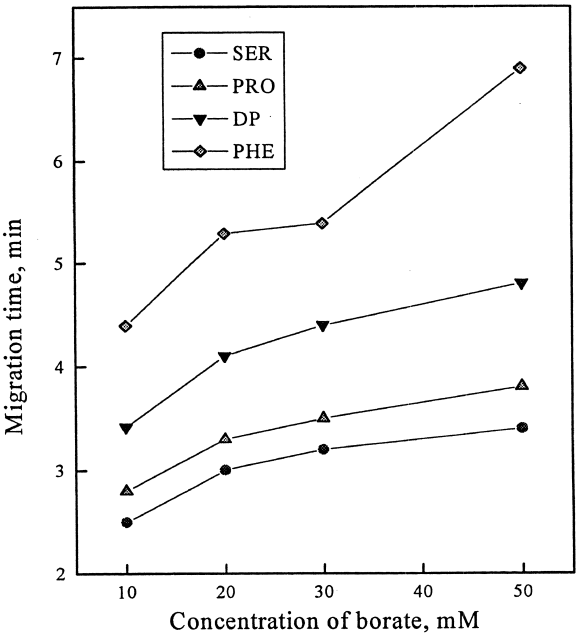

Fig. 1. The effect of borate concentration on migration time of examined analytes. Conditions: pH 9.2; fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 25 kV, detection UV at 214 nm. Analyte concentration: 1 m*M* for each analyte.

The borate concentration also affected the measurement efficiency. Changing the buffer concentration from 10 to 20 m*M* increased efficiency, especially for phenylalanine. Further increase in the buffer concentration to 30 m*M* showed further efficiency improvement for the analytes having a short migration time, but a distinct efficiency reduction for the last two analytes, as a result of increasing migration time and peak shape distortion. At a 50-m*M* concentration, the separation efficiency for phenylalanine was reduced while that for dopamine continued to improve.

With 10% methanol added to the background electrolyte, resolution of short migration time analytes was greatly enhanced, while for dopamine and phenylalamine separation was worse. The addition of 20% methanol reduced the resolution of short migration time analytes and caused a slight resolution improvement for dopamine and phenylalanine.

Table 1
Protonation constants of selected neurotransmitters [22]

Analytes	Protonation constants (pK_a)	Temperature (°C)
Adrenaline	10.02	25
	8.78	25
	2.58	25
Dopamine	11.9	20
	10.4	20
	8.9	20
Dihydroxyphenyloalanine	11.9	20
	10.4	20
	8.9	20
Ephedrine (+)	9.71	25
Ephedrine (−)	9.57	25
Phenylalanine	9.11	25
	2.18	25
Noradrenaline	13.0	25
	9.68	25
	8.63	25
	3.30	25
Serotonine	10.56	20
	9.42	20

3.2. Effect of using different cyclodextrins

Chiral selectors such as unsubstituted α-, β-, γ-cyclodextrins, substituted methyl-, dimethyl-, trimethyl- and carboxymethyl- β-cyclodextrins, hydroxy-propyl- α-, β- and γ-cyclodextrins and succinyl-β-cyclodextrin were examined in this study.

The effect of different concentrations, in the range from 3 to 20 m*M*, for unsubstituted α-, β- and γ-cyclodextrins was examined. The addition of α-CD, with the smallest hydrophobic cavity, to the background electrolyte did not substantially change the migration times. The largest changes in migration time were observed when α-CD was used for phenylalanine. The migration time was distinctly shortened. The phenylalanine was in anionic form (frontal peak broadening), hence shortening of its migration time suggested complex formation with α-CD that partly screened the negative charge of analyte.

The addition of β-CD increased the migration time of analytes, however, no specific interactions or analyte migration order change after its addition to the background electrolyte was observed. The effect on migration time of added β-CD was also investigated at pH 8.3. Reduction of the pH value to 8.3, while using the concentration of 20 m*M* β-CD, resulted in shortening migration times for all analytes. The observed effect was caused by inclusion complex formation with β-CD, and resulted in screening of charges of analytes and limiting the effect of surroundings on their mobility. The smallest migration increase was observed for neutral and positive analytes and the distinct changes occur for negative analytes, for which increasing the pH value in the background electrolyte without cyclodextrin also enlarges their migration time. Addition of the β-CD to the background electrolyte at pH 8.3 caused differentiation of serotonin and ephedrine mobility that enables their separation.

Application of γ-cyclodextrin with the biggest cavity, as a chiral selector, reduced differentiation of analytes and caused deterioration in their separation. Increase of migration times for positive and neutral analytes present in the solution caused the migration time for phenylalanine to decrease, most likely due to complex formation with neutral γ-CD.

The effect of all unsubstituted cyclodextrins on separation was examined at the applied voltages of 15, 20 and 25 kV. It can be concluded that application of unsubstituted cyclodextrins as chiral selectors did not allow satisfactory enantiomer separation to be obtained. In earlier studies, unsubstituted β- and γ-cyclodextrins were used by Szeman and Ganzler [4] for the separation of adrenaline enantiomers, Quang and Khaledi [3] for separation of adrenaline, noradrenaline, propranolol and isopropranolol, and Leroy et al. [2] for enantiomeric separation of norephedrine and phenylalanine. In all these cases, however, incomplete enantiomeric separation was observed.

Similarly, application of hydroxypropylated α-, β- and γ-cyclodextrins in the concentration range 3–100 m*M* at applied voltages of 15–25 kV did not allow successful enantiomeric separation of the neurotransmitters examined.

The addition of neutral, substituted cyclodextrins, such as M-β-CD, DM-β-CD and TM-β-CD, had some effect on the separation and chiral resolution of examined analytes. The effect of substituted CD,

added to the background electrolyte at pH 9.2, was investigated in the concentration range from 10 to 100 m*M*. Addition of M-β-CD caused the migration time for all analytes to increase. Furthermore, when the concentration of M-β-CD exceeded 75 m*M*, the resolution of ephedrine, propranolol, dopamine and adrenaline decreased. Complex formation with cyclodextrin caused the analytes' mobility differences to increase. Moreover, increasing the M-β-CD concentration decreased EOF and caused the migration time for all analytes to increase, most likely due to an increase in viscosity of the carrier electrolyte. Similar effects were observed for DM-β-CD and TM-β-CD. Application of methyl derivatives did not bring about a change in the order of analyte migration in comparison with the background electrolyte with or without unsubstituted cyclodextrins. In these circumstances, good enantiomer separation was not observed.

Substituted carboxymethyl-β-CD (CM-β-CD) was the first chiral compound in our series which was charged. CM-β-CD can be used in both the uncharged and charged form. Below pH 5 it is uncharged, while above pH 5 the carboxylic group becomes negatively charged due to deprotonation [12]. CM-β-CD had previously been used for the separation of ephedrine and propranolol enantiomers [11–15]. In this work charged CM-β-CD was employed to separate selected neurotransmitters at pH values from 7.1 to 9.2. The effect of applied voltage on migration time has also been studied and the influence of different concentrations of CM-β-CD (in the range from 3 to 20 m*M*) was examined.

Addition of CM-β-CD to the background electrolyte at pH 9.2 affected the order of migration of all analytes. The initial migration times for serotonine, ephedrine, propranolol, dopamine, adrenaline, noradrenaline, phenylalanine and DOPA were observed to change immediately when the concentration of CM-β-CD was 3 m*M*. As the concentration of the selector was increased to 20 m*M*, migration followed the order: serotonin, dopamine, adrenaline, noradrenaline, phenylalanine, ephedrine, propranolol and DOPA. All analytes showed longer migration times in the presence of CM-β-CD. Fig. 2 presents the effect of CM-β-CD concentration on migration time of the various neurotransmitters.

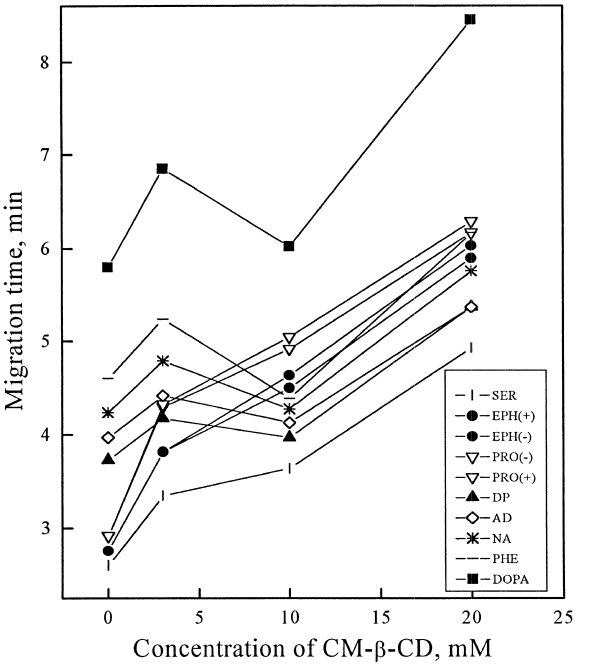

Fig. 2. The effect of CM-β-CD concentration on migration time of neurotransmitters. Conditions: background electrolyte 20 m*M* sodium tetraborate, pH 9.2; fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 25 kV, detection UV at 214 nm. Analyte concentration 1 m*M* for each analyte.

In alkaline solutions CM-β-CD is negatively charged. Hence, due to its interaction with analytes, observed migration times increased. As the concentration of CM-β-CD was increased to 10 m*M*, improved separation of ephedrine and propranolol enantiomers was observed. While at 20 m*M* CM-β-CD the mobility of adrenaline and dopamine decreases and separation decays. Increase in the CD concentration up to 20 m*M* substantially increased phenylalanine migration time and, as a consequence, phenylalanine and propranolol separation deteriorated. This effect is probably the result of specific phenylalanine adjustment to the CM-β-CD cavity. At pH 8.3 the increased cyclodextrin concentration caused similar effects to these observed at pH 9.2. However, decrease of pH allowed us to obtain adrenaline and phenylalanine enantiomeric separation (Fig. 3). The order of migration of analytes was observed as a consequence of changes of pH value, especially for phenylalanine. At pH 9.2 phenylal-

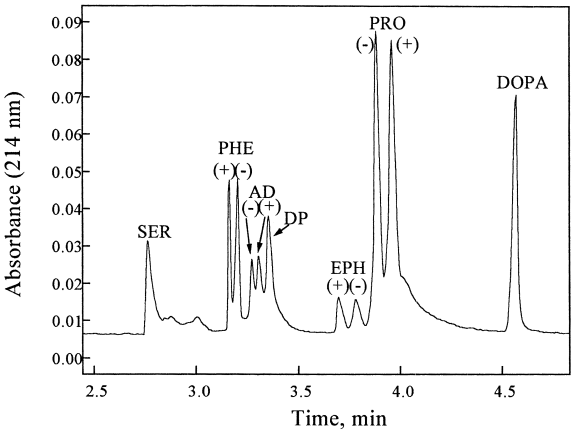

Fig. 3. Electropherogram of the mixture of neurotransmitters. Conditions: background electrolyte 20 m*M* sodium tetraborate, 20 m*M* CM-β-CD, pH 8.3; fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 30 kV, detection UV at 214 nm. Sample concentration 1 m*M* each of analyte.

anine is in its anionic form. As the pH value was decreased to 8.3, phenylalanine became partly protonated, the mobility increased and as a consequence, phenylalanine migration time decreased. Further pH decrease caused increasing migration time for all analytes as a result of decrease in electroosmotic flow. Application of background electrolyte at pH 7.1 with addition of 20 m*M* of CM-β-CD did not allow us to obtain phenylalanine and adrenaline enantiomeric separation.

Another cyclodextrin, which affected the migration times of the neurotransmitters, was Succ-β-CD. Succinyl-β-CD, again a negatively charged CD-derivative, was used at a concentration of 20 m*M*. Addition of 20 m*M* Succ-β-CD to the background electrolyte completely ruins separation, while a decrease in the concentration to 10 m*M* provided incomplete separation without chiral separation.

It was then concluded that addition of 20 m*M* CM-β-CD to the borate electrolyte was optimal for separation of neurotransmitters. At this concentration of CM-β-CD four ephedrine enantiomers separated (Fig. 4). Baseline adrenaline and propranolol separation was achieved at 20 kV, but required a lower pH value of 7.5 (Fig. 5). This result was obtained at the expense of loss of phenylalanine enantiomer separation and substantial increase of migration times of all analytes. The concentration detection limits ($S/N=3$) in these conditions were in the range

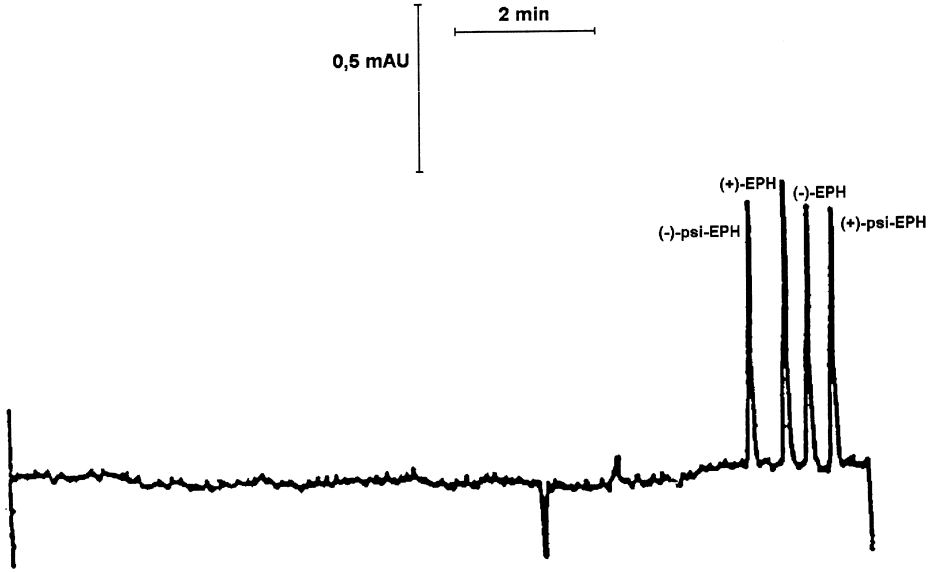

Fig. 4. Electropherogram of the ephedrine enantiomers. Conditions: background electrolyte 20 m*M* sodium tetraborate, 20 m*M* CM-β-CD, pH 7.5; fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 20 kV, hydrostatic injection 5 s, detection UV at 214 nm.

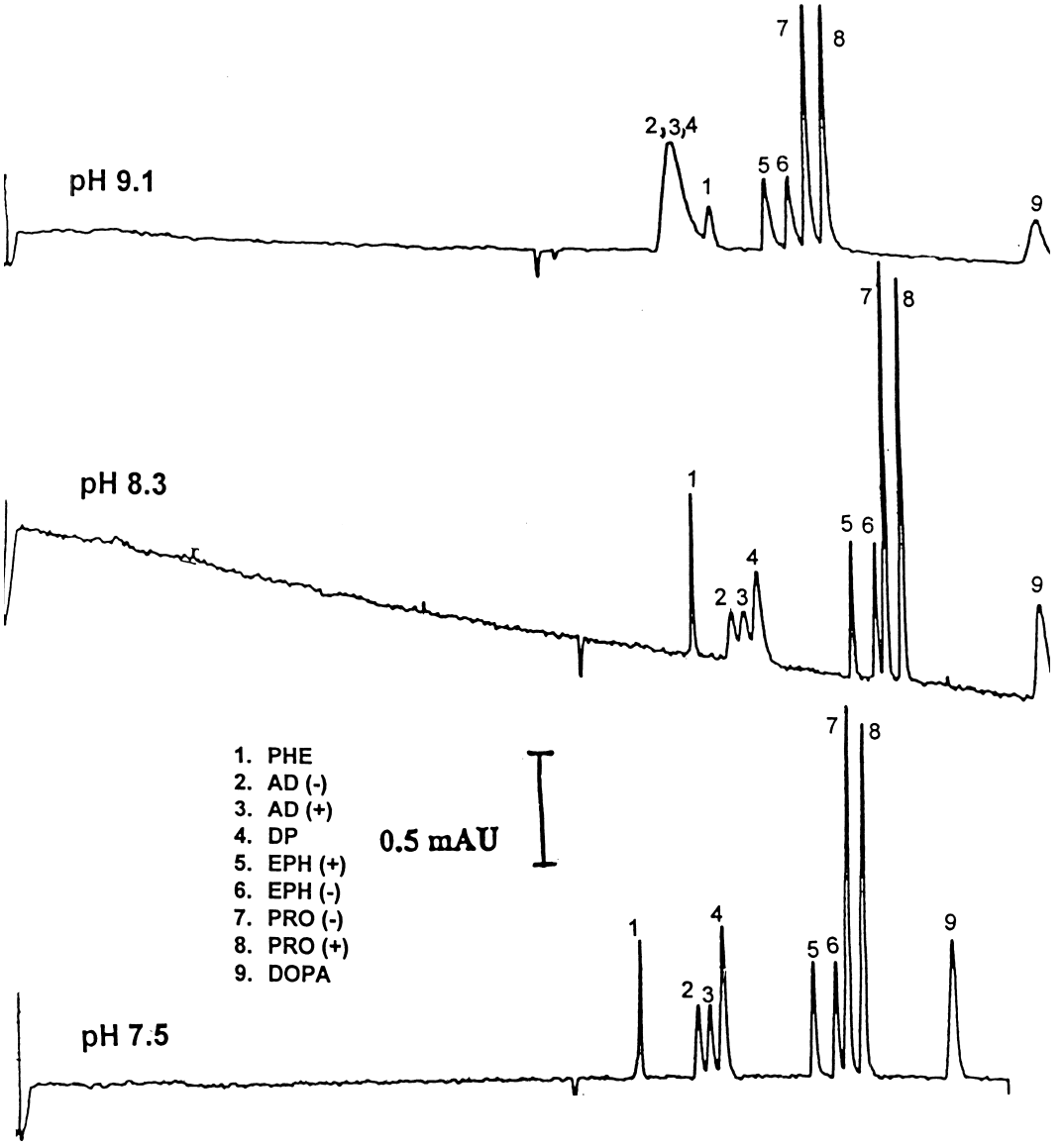

Fig. 5. The effect of pH on the separation of neurotransmitters. Conditions: background electrolyte 20 m*M* sodium tetraborate, 20 m*M* CM-β-CD, fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 20 kV, hydrostatic injection 5 s, detection UV at 214 nm.

from 40 μ*M* for propranolols to 0.2 m*M* for adrenalines. The observed sensitivities (peak height) varied from 0.4 for adrenalines to 2.3 mAU mM^{-1} for propranolols. The efficiency, expressed as a number of theoretical plates, varied from 12 980 for phenylalanine to 3170 for DOPA.

3.3. Determination of neurotransmitters in biological samples

An examination of the effect of common interferences present in blood serum was also carried out. The effect of the presence of 0.15 *M* NaCl, 2 m*M*

$CaCl_2$, 1 m*M* $MgCl_2$, 5 ppm Fe(III), 5 ppm Cu(II), 10 and 30 mg l^{-1} albumin in the sample was examined. Results obtained indicated that common interferences do not significantly affect the signal of neurotransmitters, except in the presence of 30 mg l^{-1} albumin, which caused an increase in signal for all analytes, especially for propranolol. We intend to study this point further in the future.

The electrophoretic separation of dopamine and adrenaline in brain tissue has been already reported [23–25]. Adrenaline, noradrenaline and dihydroxyphenylalanine were also determined by CE in urine samples [6]. Paquette et al. [21] used laser-induced fluorescence detection with a KrF excimer laser to investigate the capillary electrophoretic profiles of human urine, saliva and serum without the need for sample derivatization. Siren and Karjalainen [1] used capillary zone electrophoresis with photodiode array detection for analysis of catechol compounds in human urine.

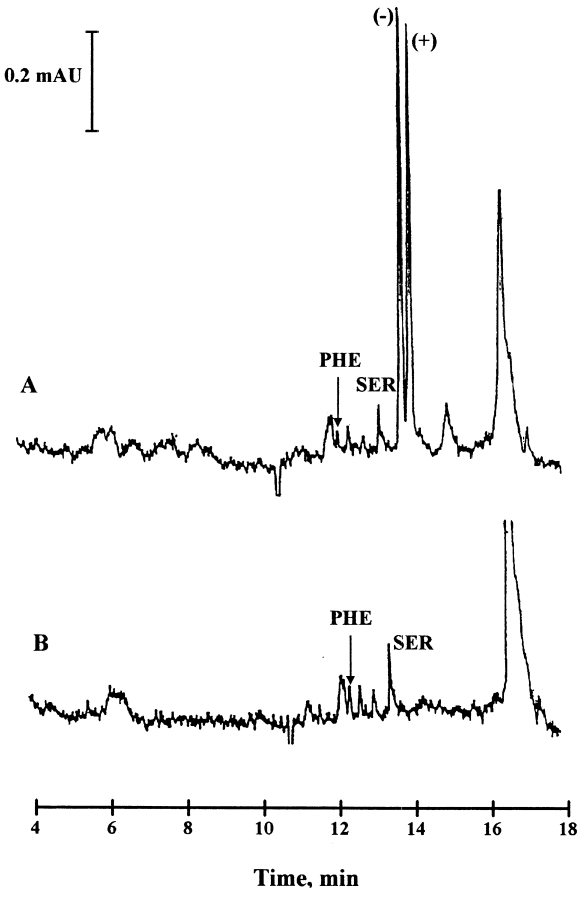

Fig. 6. Electropherograms of human cerebrospinal fluid obtained at 20 kV with buffer consisting of 20 m*M* sodium tetraborate and 20 m*M* CM-β-CD (pH 7.5). Sample filtered (0.45 μm) and hydrostatic injected 5 s. Detection UV at 214 nm. (A) Sample spiked with NA. (B) Sample without NA added.

A preliminary application of developed separation method to physiological samples was made. The electropherogram of cerebrospinal fluid obtained in this work with 20 m*M* sodium tetraborate electrolyte containing 20 m*M* CM-β-CD at pH 7.5 is shown in Fig. 6. Pressure driven hydrodynamic injections of sample were employed for 5 s, and voltage of 20 kV was applied. The samples were only pretreated by filtration through the 45-μm filter before injection. The serotonine and phenylalanine were found at the levels of 319 and 190 μg ml^{-1}, respectively, which are means of four injections.

Using the same conditions, commercial pharmaceutical preparations Gripex and Nurofen were examined for the presence of (+)-Ψ-ephedrine (Fig. 7). Powdered tablets were dissolved in Milli-Q water, filtered (45 μm) and hydrostatically injected into the capillary without any other pretreatment. Preliminary quantitative estimation of results provided a level of (+)-Ψ-ephedrine about 20% lower than labeled. More detailed quantitative work on the determination of the examined analytes in several commercial preparations is in progress.

4. Conclusions

The examination of a wide spectrum of cyclodextrins allowed optimization of CE conditions for separation of serotonin, dopamine, adrenaline, ephedrine, propranolol, phenylalanine and dihydroxyphenylamine in a single run, including separation of most existing enantiomers. The best results for separation of neurotransmitters and their enantiomers were obtained using 20 m*M* carboxymethyl-β-CD in borate buffer of pH 7.5 with 5-s hydrostatic injection and an applied voltage of 20 kV. The concentration detection limits ($S/N=3$) were at the level of μg ml^{-1}. The developed method was employed for preliminary determination of these analytes in biomedical and pharmaceutical samples.

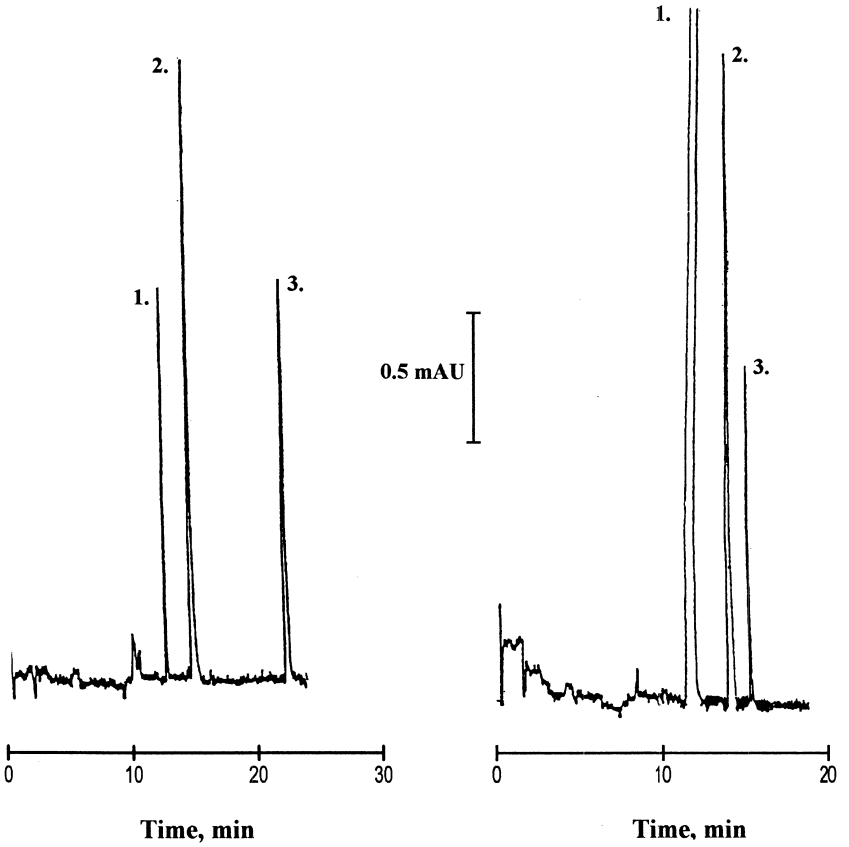

Fig. 7. Electropherograms of pharmaceuticals: Gripex (right) and Nurofen (left). Conditions: background electrolyte 20 m*M* sodium tetraborate, 20 m*M* CM-β-CD, pH 7.5; fused-silica capillary 75 cm (65 cm to the detector)×50 μm I.D., voltage 20 kV, hydrostatic injection 5 s, detection UV at 214 nm. Peaks: 1, unknown; 2, (+)-ι-ephedrine; 3, unknown.

Acknowledgements

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