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Capillary zone electrophoretic determination of some drugs against Alzheimer's disease

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Abstract

A new capillary zone electrophoresis (CZE) method for the determination of tacrine (THA), 7-methoxytacrine (7-MTHA) and their basic metabolites (THAm, 7-MTHAm) in pharmaceutical and biological samples (urine and serum) was developed. Separation of all compounds by CZE was carried out using a 46.6 cm untreated fused-silica capillary applying 20 kV separation voltage using 50 m*M* phosphate buffer of pH 2.8 for THA and THAm and of pH 7.8 for 7-MTHA and 7-MTHAm as background electrolyte (BGE). Detection was carried out at 240 nm (THA and THAm) and 248 nm (7-MTHA and 7-MTHAm). THA and THAm were separated in less than 4 min while 7-MTHA and 7-MTHAm were separated in less than 7 min. The detection limits ($S/N=3$) obtained were 3 ppb for THA and 4 ppb for 7-MTHA in aqueous solutions; 50 ppb for THA and 47 ppb for 7-MTHA for the determination in urine (diluted 1:10); 52 ppb for THA and 56 ppb for 7-MTHA, in deproteinized serum samples. The methods are suitable for therapeutic drug monitoring of the drugs.

Keywords: Tacrine; Methoxytacrine

1. Introduction

Alzheimer's disease (AD) is a slow, progressive neuropsychiatric illness of unknown etiology [1], frequently it is related to depletion of central cholinergic activity. AD is principally characterized by a progressive loss of memory and intellectual function. In 1986, Summers et al. [2] described the results of a clinical study in which 1,2,3,4-tetrahydro-9-aminoacridine (THA) was administered orally in combination with lecithin as an acetylcholine precursor to treat patients with AD. The results of this study were positive and encouraging. On 9 September, 1993, the US Food and Drug Administration (FDA) announced that THA (tacrine, Cognex/Parke-Davis) had become the first drug specifically approved for the treatment of AD [3]. THA is a potent inhibitor of both acetylcholinesterase and butyrylcholinesterase. It was reported that THA seems to induce liver damage in Alzheimer patients [4,5], which may preclude its use as a safe drug in treatment of AD. Another structural analogue, 9-amino-7-methoxy-1,2,3,4-tetrahydroacridine (7-methoxytacrine, 7-MTHA) was developed as a less toxic and pharmacologically equally effective derivative of THA [6,7].

Several high-performance liquid chromatographic (HPLC) procedures for the determination of THA in biological samples have been reported. These methods employ ultraviolet (UV) [8–11], fluorescence [12,13] and electrochemical [14] detection with a detection limit of 0.2–100 ng/ml of plasma. Among

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these methods, application of capillary zone electrophoresis (CZE) has not been published so far though these species may form ions in solution. It is important that a sensitive and precise assay be available for the quantification of this drug so that dosing requirements can be assessed. Capillary electrophoresis (CE) appears a suitable candidate method for separating tacrine and its derivatives (metabolites); in this report we summarize our results regarding THA, 7-MTHA and their metabolites and this electrodriven technique in urine and serum.

2. Experimental

2.1. Reagents

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine monohydrate, THA), tacrine metabolite (9-amino-1-hydroxy-1,2,3,4-tetrahydroacridine maleate, THAm), 7-methoxytacrine (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine lactate, 7-MTHA) and 7-methoxytacrine metabolite (9-amino-7-hydroxy-1,2,3,4-tetrahydroacridine chloride monohydrate, 7-MTHAm) were supplied by the Department of Toxicology, Military Medical Academy (Hradec Králové, Czech Republic). Lyophilized urine was from Chemie Biotrol (France). Phosphoric acid and sodium hydroxide were of analytical grade (Lachema, Chemapol, Czech Republic). Mesityl oxide (MSO) used as a marker (Fluka, Buchs, Switzerland) and standard pH buffers were from the Institute of Serum and Vaccines (Prague, Czech Republic). Three times distilled water used to prepare all the solutions was obtained from a quartz distillation stand (Heraeus Quarzschmelze, Hanau, Germany).

2.2. Apparatus

A Beckman CZE (Model P/ACE) System 5500 (Palo Alto, CA, USA) equipped with a diode array detection (DAD) system, automatic injector, a fluid-cooled column cartridge and System Gold Data station was utilized for all experiments. Fused-silica capillary tubing of 46.6 (39.0 cm length to the detector)×75 μm I.D. was used. Spectrophotometric measurements were done on a UV-2 Quart UV–Vis Unicam spectrometer (Cambridge, UK). A Radelkis OP-208 Precision Digital pH-meter (Budapest, Hungary) and a Radelkis pH sensitive, combined glass electrode were used for the pH measurements.

2.3. CE measurements and procedure

CZE separations were carried out at 25°C. The solution of a 50 m*M* phosphate buffer used throughout this study, was prepared daily, filtered through 0.2-μm nylon filters and submitted to Ultrasonic Cleaner (Branson, USA). The pH was adjusted with diluted NaOH and/or HCl solutions. The capillary inlet and outlet vials were replenished after every five injections. The capillary was washed daily for 10 min with 0.1 *M* NaOH, 10 min with deionized water and 10 min with run buffer. In each analysis prewash of 2 min with 0.1 *M* NaOH and 2 min with background electrolyte (BGE) was utilized. Absorbance was monitored at 200 nm. The electroosmotic flow (EOF) was determined using 0.1% (v/v) MSO injected under the same conditions as for the separation of samples. In the preliminary experiments hydrodynamic injection 5 s was utilized. Then, electrokinetic injection was applied in the rest of the experiments: 80 s, 10 kV for aqueous solutions, while for urine and serum samples 10 s, 10 kV was used for THA and THAm and 50 s, 7 kV for 7-MTHA and 7-MTHAm.

2.4. Sample preparation

Urine samples were diluted taking 100 μl to 1 ml of water. Serum (100 μl) was deproteinized by mixing with 150 μl acetonitrile for 15 s and then the mixture was centrifuged for 1 min at 15 000 *g* and supernatant taken for the analysis.

3. Results and discussion

The absorption spectra of the compounds studied are shown in Fig. 1. Detection at 240 nm for THA and its metabolite and 248 nm for 7-MTHA and its metabolite were used as the most sensitive wavelengths for the determination. The molecular structures of THA, 7-MTHA and their metabolites can be seen in Fig. 2. The cationic behavior of these compounds in acid solution is due to the presence of

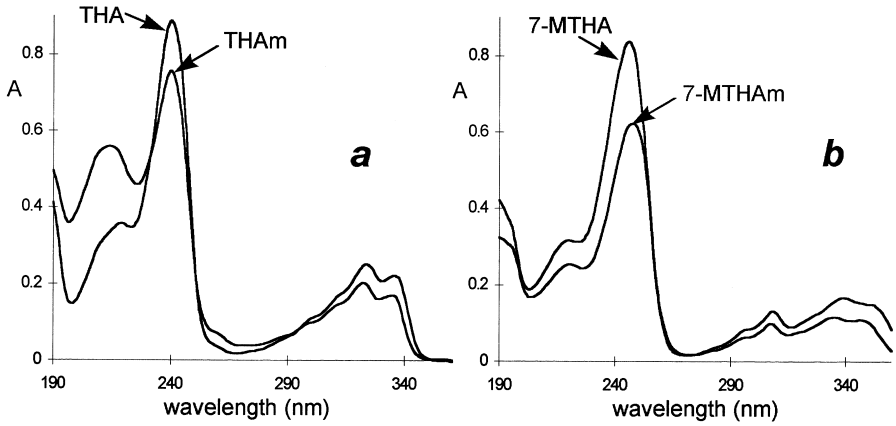

Fig. 1. Absorption spectra of anti-Alzheimer drugs. (a) THA and THAm (5.4 ppm), (b) 7-MTHA and 7-MTHAm (5.2 ppm) in aqueous solution.

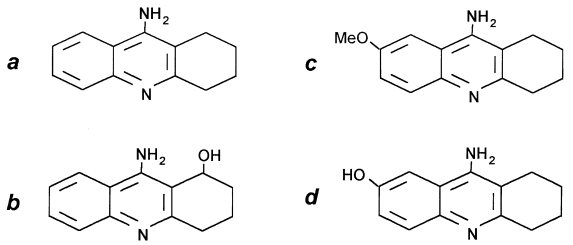

Fig. 2. Molecular structures of the anti-Alzheimer drugs. (a) THA (1,2,3,4-tetrahydro-9-aminoacridine); (b) THAm (9-amino-1-hydroxy-1,2,3,4-tetrahydroacridine); (c) 7-MTHA (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine); (d) 7-MTHAm (9-amino-7-hydroxy-1,2,3,4-tetrahydroacridine).

the amine group in the molecule which can be protonated. The effective mobility as a function of pH confirms cationic behavior over almost all the pH range studied (Fig. 3). Considerable decrease of the effective mobility can be observed for THA, THAm and 7-MTHA after pH 8 due to the dissociation of protonated amino group. Effective mobility of 7-MTHAm decreases for pH higher than 6 due to the dissociation of protonated amino group and because of the dissociation of the hydroxyl group which imparts zwiterionic behavior and/or negative charge at very high pH. Satisfactory resolution of THA and THAm was achieved over all the pH range studied

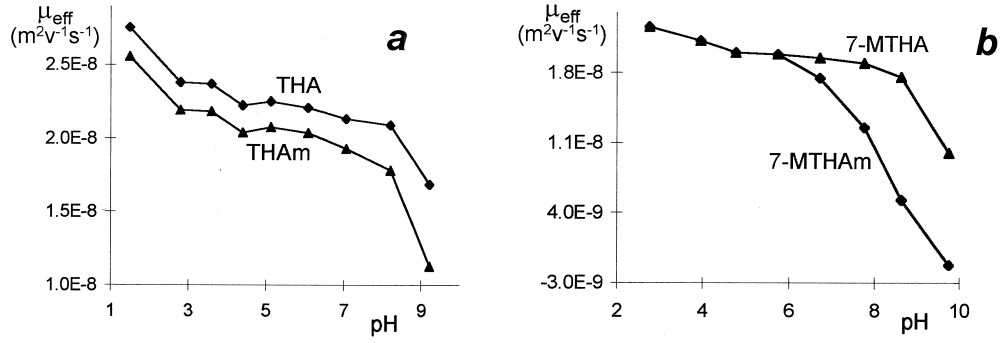

Fig. 3. Effective mobility as a function of pH. (a) THA (26.7 ppm) and THAm (29.0 ppm); (b) 7-MTHA (25.3 ppm) and 7-MTHAm (27.4 ppm). A 0.1% MSO solution was used as a neutral marker for the EOF. Separation conditions: fused-silica capillary 46.6 cm total length (39.0 cm length to the detector), 50 m*M* phosphate buffer as the BGE, separation voltage 20 kV (negative polarity at the detection side), temperature 25°C, hydrodynamic injection 5 s (0.5 p.s.i.). Detection wavelengths: 240 nm for THA and 248 nm for 7-MTHA.

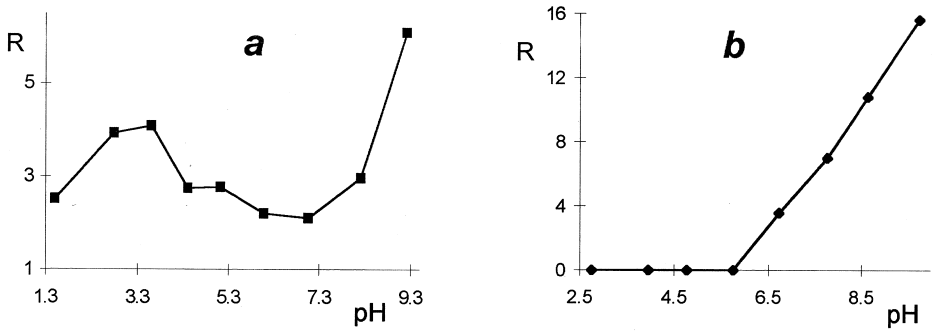

Fig. 4. Anti-Alzheimer drugs resolutions as a function of pH. (a) Resolution of THA and THAm; (b) resolution of 7-MTHA and 7-MTHAm. Other conditions as in Fig. 3.

(Fig. 4). The value of pH 2.8 was selected as optimal because high resolution can be reached and over-heating was avoided. On the other hand 7-MTHA and 7-MTHAm were resolved well only at pH higher than 6.5. The value of pH 7.8 was chosen as optimal because of good resolution obtained at this pH and no over-heating was observed.

Different modes of injection were tested (Fig. 5) in order to obtain the highest sensitivity and the best selectivity of the determination. The compounds were loaded into the capillary by an electrokinetic injection (80 s, 10 kV) instead of hydrodynamic injection (20 s, 0.5 p.s.i.; 1 p.s.i.=6894.76 Pa). Increase of the time of hydrodynamic injection, increases the peak height, however, peak broadening appears for injections larger than 40 s and the resolution is lost. Therefore, in our work we have used electrokinetic injection. Because the positive ions migrate faster than neutral and anionic compounds, this kind of injection generates cation stack-

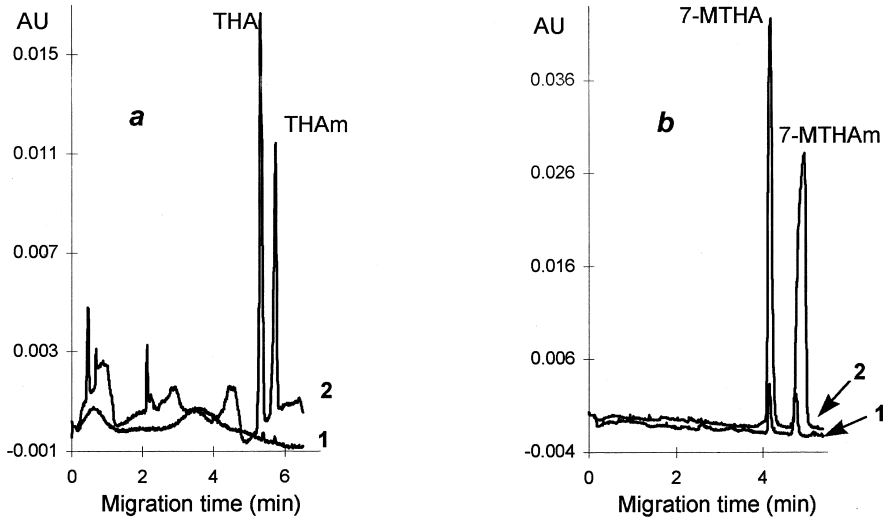

Fig. 5. Comparative electropherograms of (a) THA (88 ppb) and (b) 7-MTHA (5.9 ppm) and their metabolites using different kind of injections. (1) Hydrodynamic injection 20 s (0.5 p.s.i.); (2) electrokinetic injection 80 s, 10 kV. Other conditions as in Fig. 3.

ing at the beginning of the capillary. As a consequence of this preconcentration, an increase of the sensitivity occurs.

3.1. Analysis of THA, 7-MTHA and theirs metabolites in aqueous solution

Calibration curves in aqueous solutions were measured for the following concentrations: 9, 18, 35, 70 and 140 ppb for THA and THAm, and 4, 13, 26, 51 and 103 ppb for 7-MTHA and 7-MTHAm. The curves were linear in the concentration range studied with a quite acceptable correlation coefficient, r^2 (see Table 1). The LOD (limit of detection) for all compounds in aqueous solution were defined at signal-to-noise ratio (S/N)=3 and with n=5 (n is the number of experiments). The LOD of 3 ppb for THA and its metabolite was reached while for 7-MTHA and its metabolite 4 ppb was achieved. The reproducibility of the migration times as 1.3% relative standard deviation (R.S.D.) was observed for n=10.

Ampoules (25 mg/1 ml) and tablets (25 mg) obtained by the Research Institute of Pharmacy and Biochemistry (Prague, Czech Republic) were analyzed with the method developed. The percent purity found for two different pharmaceutical products were found in the acceptable range (>98%), the results are summarized in Table 2.

3.2. Analysis of THA, 7-MTHA and their metabolites in urine

One of the great advantages of CZE in routine analysis is the simplicity of the sample treatment. Sometimes even no sample pretreatment is necessary or only dilution of the urine sample is necessary. Direct analysis of urine samples can be difficult due to the high concentration of salts and many other compounds. In order to minimize these effects on the resolution because of the variation in the matrix composition urine can just be diluted and then analyzed. The procedure can be applied whenever the sensitivity of the determination is sufficient. Therefore, tenfold urine dilution was applied in all the experiments. Examples of the separation and determination of the compounds studied in urine are shown in Figs. 6 and 7. A good specificity of the determination was observed because other peaks from the urine (see blank) did not interfere around the migration times of the drugs monitored. Urine and serum, in general, have less interferences in the cationic compared to the acidic compounds. It is well known that the BGEs with a high ionic strength have the advantage of yielding better resolution and producing sharper peaks [15]. This is probably a consequence of the decrease of the diffusion. Also lower protein adsorption was observed in uncoated capillaries at high ionic strength. For these reasons it is recommended to use high ionic strength buffers when analyzing samples derived from serum or urine even though these buffers slow down the analysis [16]. For that reason a 300 m*M* phosphate buffer was used as BGE for urine and serum sample determinations in this work.

Known amounts of THA, 7-MTHA and their metabolites were used to spike the tenfold diluted urine and the calibration curves were measured. Linear equations were found in the following concentration range: 0.073 to 2.23 ppm for THA and its metabolite and 0.071 to 1.2 ppm for 7-MTHA and its

Table 1
Calibration curve equations of tacrine derivatives in aqueous solutions

	Slope	Intercept	r^2	LOD (ppb) (S/N=3)
THA	0.0228	−0.0561	0.998	3
THAm	0.0177	0.0792	0.999	3
7-MTHA	0.0032	0.0099	0.999	4
7-MTHAm	0.0036	−0.0060	0.999	4

LOD=Limit of detection.

Table 2
Results of analysis of the content of 7-MTHA in different medicaments

	Declared (mg)	Found[a] (mg)±σ	Purity % (rel.)
Ampoules[b]	25	24.69±0.12	98.8
Tablets[b]	25	24.80±0.09	99.2

[a] Mean of three analyses.
[b] Obtained by the Research Institute of Pharmacy and Biochemistry (Prague, Czech Republic), not available commercially.

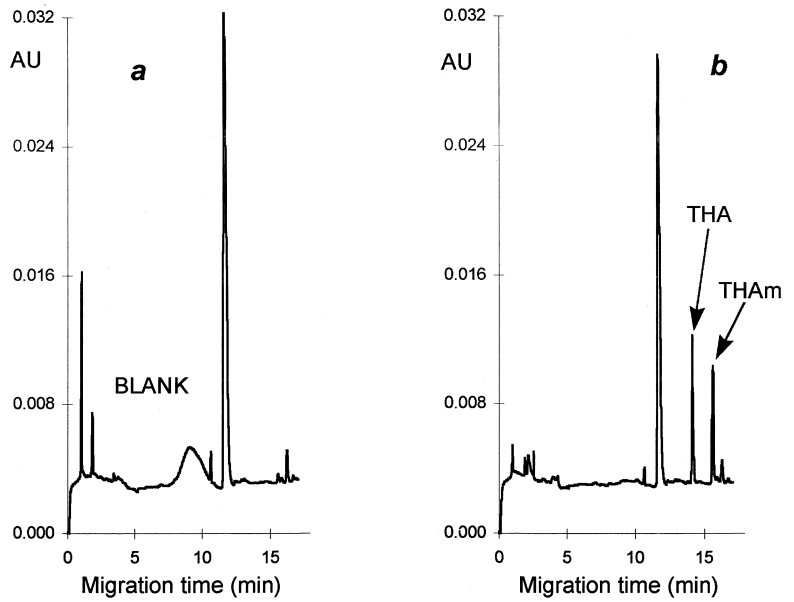

Fig. 6. Electropherogram of (a) urine blank and (b) urine sample spiked with THA and THAm (2.12 ppm). 300 m*M* phosphate buffer, pH 2.8, electrokinetic injection 10 s, 10 kV. Urine was diluted (1:10) before the analysis. Other conditions as in Fig. 3.

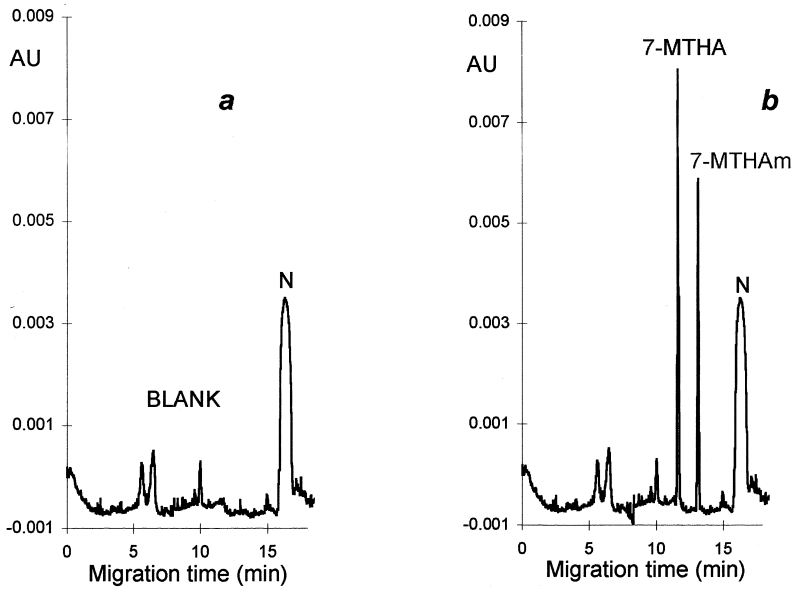

Fig. 7. Electropherogram of (a) urine blank and (b) urine sample spiked with 7-MTHA and 7-MTHAm (1.18 ppm), N means the neutral compound(s) originated from the urine matrix. 300 m*M* phosphate buffer of pH 7.8, electrokinetic injection 50 s at 7 kV. Urine was diluted (1:10) before the analysis. Other conditions as in Fig. 3.

Table 3
Calibration curves equations of the drug determination in urine

	Slope	Intercept	r^2	LOD (ppb) ($S/N=3$)
THA	0.424	0.095	0.993	50
THAm	0.355	0.107	0.992	50
7-MTHA	1.941	−0.014	0.999	47
7-MTHAm	1.980	0.077	0.999	47

metabolite (Table 3). Acceptable correlation coefficients r^2 were observed with LOD defined at $S/N=3$ and $n=5$. Fifty ppb for THA and THAm was reached while 47 ppb for 7-MTHA and 7-MTHAm was achieved. The reproducibility of the migration times were 1.53% R.S.D. for $n=10$. Recovery values of 99.2 (THA), 101 (THAm), 99.5 (7-MTHA) and 98.9% (7-MTHAm) for urine were found. We suggest that near 100% recovery is also valid for serum. Typical electropherogram for analysis of urine from a dog which was receiving a single dose of 10 mg/kg of 7-MTHA can be seen in Fig. 8.

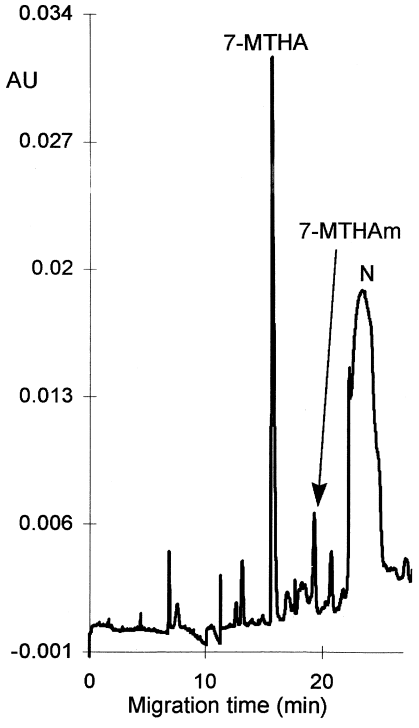

Fig. 8. Electropherogram of lyophilized urine sample of a dog which was receiving a simple dose of 10 mg/kg of 7-MTHA, diluted 1:10, N means the neutral compound(s) originated from the urine matrix. Obtained under optimal conditions.

3.3. Analysis of THA, 7-MTHA and their metabolites in serum

Serum samples contain high concentrations of proteins and ions which interfere in the analysis of small molecules. Traditional solvent extraction, of course, eliminates the problem of proteins and ions presented in the serum samples. However, it requires several additional steps and increases the analysis time. Precipitation of serum proteins with acetonitrile has been applied many times in the determination of drugs by CE [17]. Pure acetonitrile is a very good deproteinization agent but the problem is that it does not conduct current. Mixtures of acetonitrile–water on the other hand, are sufficiently conductive. Shihabi [18] determined that acetonitrile–water (60:40) was optimal for CE studies. The acetonitrile not only removes the serum proteins but also increases the peak heights of some compounds. Examples of the separation of anti-Alzheimer drugs in serum by the CE method developed are shown in Fig. 9.

4. Conclusions

A simple, sensitive and fast CZE method for the analysis of THA, 7-MTHA and their metabolites in aqueous, urine and serum samples was developed. Analysis of the pharmaceutical formulations can be made directly, while tenfold dilution was applied in the case of urine samples. Deproteinization with acetonitrile was used in the case of serum analysis. CZE offers advantages over the other methods in terms of short analysis time, low cost and simple pretreatment of the samples. Therapeutical drug monitoring of the anti-Alzheimer drugs studied can be easily done applying the procedures developed.

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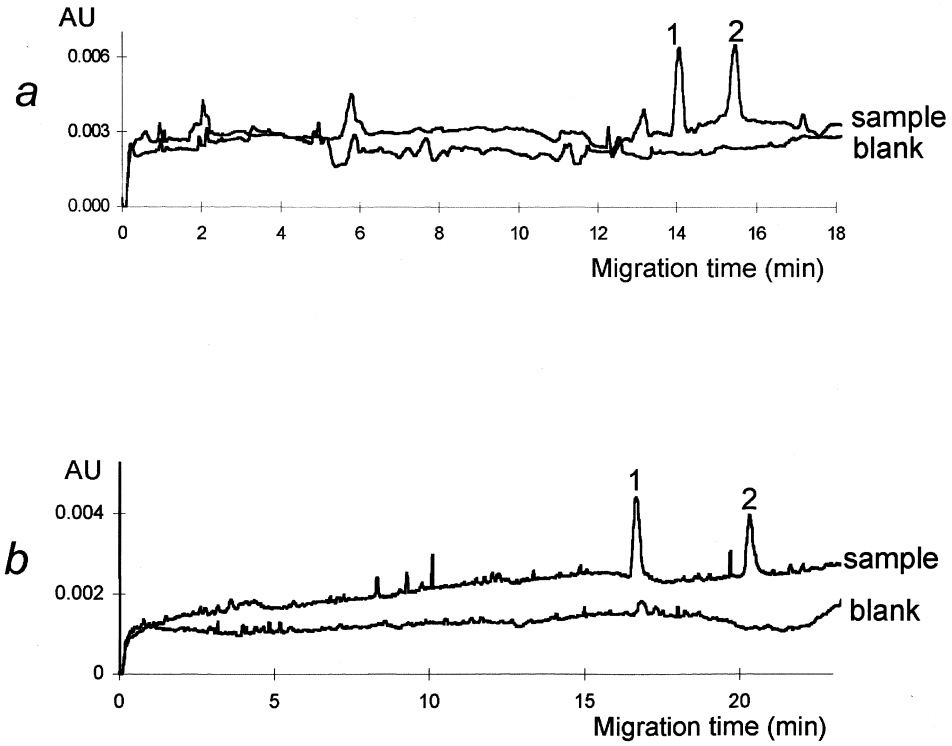

Fig. 9. Electropherograms of deproteinized serum sample spiked with (a) 1=THA and 2=THAm (225 ppb); (b) 1=7-MTHA and 2=7-MTHAm (1.561 ppm). Obtained under optimal conditions.

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