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Separation of tricyclic antidepressants by capillary zone electrophoresis with *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine (TMBD) as an effective electrolyte additive

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Abstract

Five tricyclic antidepressants (TADs), desipramyne, nortriptyline, imipramine, doxepin and amitriptyline, were separated by using the *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine (TMBD) as additive in the background electrolyte solution. Because the tricyclic antidepressants are similar in structure, mass and pka values, their separation, by capillary zone electrophoresis, requires the careful manipulation of parameters, such as the pH and the composition of the electrolyte solution. As basic drugs, the TADs interact with the silanol groups on the capillary wall giving rise to peak broadening and asymmetry, non reproducible migration times and failing in selectivity. Different concentrations of TMBD (40, 60, 100 and 150 mM) were used at pH 9.5, but only a 100 mM TMBD allowed a good separation and a high efficiency for all the TADs. At this pH the separation was not possible without additive. This result is due to the reduced electroosmotic flow whose mobility is at a value of 10^{-9} m^2 V^{-1} s^{-1}.

Keywords: Tricyclic antidepressants; *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine; Capillary zone electrophoresis

1. Introduction

Tricyclic antidepressants (TADs) is an archaic term referred to the structure of a group of drugs commonly used in therapy for the depression desease. The term cannot be referred to the mechanism of action, as new agents, with different structure, block the biogen amine reuptake like TADs do [1].

In this paper five TADs with similar structure, desipramyne and nortriptyline (secondary amines), and imipramine, doxepin and amitriptyline (terziary amines) have been considered.

Several techniques have been employed to detect the TADs in biological samples: spectrophotometry, gas-chromatography (GC), radio-

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immunoassay (RIA), and reversed phase high performance liquid chromatography (RP-HPLC).

As a relationship between the plasmatic concentration and the clinical effects occurs, it is important to know the quantity of TADs in plasma.

Spectrophotometric method is sensitive only when TADs are present at high concentration, close to the toxic dose, [2] and does not reveal low concentrations which are used in therapy.

To detect small amounts of TADs an electron capture gas-chromatography method is preferred [3]. In fact, this technique can detect up to 1 ng ml^{-1} of TADs. It is a fast technique as the analysis time range between 40 and 60 min, which is a normal length of time occurred in GC. Unfortunately, this technique is not enough selective towards TADs and requires long preliminary operation procedures. For this reason the GC is employed in pharmacokinetics. Besides, the GC requires long extractions and derivatisation, which take a long time that it is not acceptable for routine analysis. Moreover, just because of the long time required, the number of samples which can be analysed daily is reduced.

Radioimmunoassay technique avoids long procedures and is highly sensitive [4]. This technique can analyse up to 40 samples in duplicate between 4 and 5 h. Unfortunately, the antiserum used in this technique reacts in a different way towards the amitriptyline and the nortriptyline. So this assay seems not suitable to identify TADs and also in this case RIA is employed in pharmacokinetic studies.

Reversed phase high performance liquid chromatography (RP-HPLC) is the most common technique. Several aliphatic amines, e.g. ammonium hydroxide [5], or propylamine [6], have been used in the eluent to avoid the interactions between the TADs and the silanol groups in the silica columns. In order to check these interactions, the retention factor of benzyltriethylamonium bromide was measured [7]. The retention factor decreases when the quantity of sample is increased. By adding *N*-ethylaminoethanol or dimethylethanolamine the effect decreases, but when triethylamine or diisopropylamine is added, the retention factor does not depend on the quantity of sample. This means that these amines compete with the basic amines towards the silanol groups. RP-HPLC has some disadvantages. One is the sensitivity as the UV detector is nor enough sensitive to reveal small concentration of TADs [2]. Other disadvantages could be the stability of the column, the toxicity of the eluents, the cost of purchase and the disposal of waste.

Lately, several methods for the identification and determination of TADs have been employed by capillary electrophoresis [8], because of its high efficiency and versatility, very low volume samples and reagents, low cost and minimisation of the environment pollution.

The analysis of TADs by CE was mainly performed in non aqueous solution. Organic solvents can affect the efficiency of the resolution for several reasons. For example, methanol causes the current to drop down when injected into the capillary because it is a non-conducting solvent. This problem could be solved by using the electrokinetic injection mode. But the electrokinetic injection is not recommended for biological samples because of a change of conductivity of the sample matrix. This problem could be avoided by making up complicated procedures due, for example, to the on-line techniques-CE [9].

TADs are basic drugs and present similar mass other than similar structure.

Due to the close mass values, chemical structure and pK_a values, their separation, by capillary zone electrophoresis, gets difficult because of the similar mobility.

Moreover, as basic drugs, TADs interact with the silanol groups on the capillary wall, which are negatively charged at the run pH (9.5). At this pH, which is below the values of the pK_as, TADs are positively charged. This behaviour gives rise to a peak broadening and lack of selectivity and efficiency. In an attempt to develop an efficient, sensitive and fast method for identifying TADs, the properties of a new additive were investigated.

In this paper *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine (TMBD) is presented as an effective running electrolyte additive for the separation of basic drugs, as TADs, in bare fused-silica capillary. The property of TMBD in masking the silanol groups on the surface of the capillary wall

and its effect on the electroosmotic flow (eof) are investigated.

This method is proposed in order to improve the recognition of TADs which is useful especially in the case of poisoning or overdose. On the other hand, the use of TMBD requires a long conditioning time (30 min) of the capillary. Moreover, the manipulation of TMBD is unwelcome and must be carefully kept under control.

2. Experimental procedures

2.1. Materials

Desipramyne hydrochloride, nortriptyline hydrochloride, imipramine hydrochloride, doxepin hydrochloride and amitriptyline hydrochloride, were obtained from Sigma (Italy).

N,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine and mesityl oxide were purchased from Fluka (Milan, Italy).

Reagent-grade phosphoric acid (PA), boric acid (BA), formic acid (FA) and HPLC-grade water were obtained from Carlo Erba (Milan, Italy).

2.2. Equipment

All experiments were performed using a Beckman P/ACE System 2100 electrophoresis equipped with a deuterium lamp with a 214 nm band pass filter and an IBM Model 55SX computer, Version 7.11 System Gold software for system control, data collection and data analysis (Beckman, Fullerton, CA, USA).

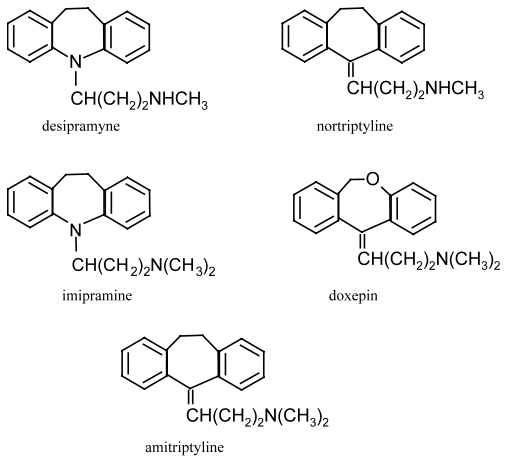

Fig. 1. Chemical structure of the tricyclic antidepressants.

Fused silica capillaries of 50 μm ID, 570 mm total length and 550 mm to the detector were purchased from Quadrex (New Haven, CT).

The capillary temperature was mantained at 25 °C by a fluorocarbon liquid circulating through the cartridge. Samples were injected into the anionic end of the capillary by applying 0.5 p.s.i. (1 p.s.i. = 6894.74 Pa) pressure for 3 s.

2.3. Capillary electrophoresis

Before use, a new bare capillary was conditioned by flushing with water (5 min), 1 M HCl (10 min), water (10 min), 1 M NaOH (15 min), water (15 min) and running buffer for 30 min. The running electrolyte solutions were: potassium borate (56 mM; pH 9.5); potassium phosphate (56 mM; pH 9.5); TMBD phosphate (20, 40, 60 mM; pH 8); TMBD phosphate (100 mM; pH 9.5). The pH was measured with a glass electrode Model HI 1131 from Hanna Instruments (Woonsocket, RI). All solutions were filtered through a Type HA 0.22 μm membrane filter (Millipore, Vimodrone, Italy) and degassed by sonication before use. The running electrolyte was renewed after three or four runs, and before each run the capillary was rinsed with the running electrolyte for 3 min.

All experiments were carried out applying a voltage of 15 kV.

3. Results and discussion

The structure of TADs is shown in Fig. 1. The two secondary amines differ just for a nitrogen atom (desipramyne) in place of a carbon atom (nortriptyline). Their molecular mass is fairly similar with only of 3 μm of a difference, and the pK_as range from 10.2 to 9.7. The three tertiary amines differ for a nitrogen atom (imipramine), an oxygen atom (doxepin) instead of a carbon atom (amitriptyline). Their molecular mass and pK_as range from 277.39 to 280.40 and from 9.0 to 9.5 respectively (Table 1) [10].

Table 1
Chemical properties of the tryciclic antidepressants

Secondary amines	Molecular weight	pK_a	Ref.
Desipramyne	266.37	10.2	[10]
Nortriptyline	263.37	9.7	
Tertiary amines			
Imipramine	280.40	9.5	
Doxepin	279.37	9.0	
Amitriptyline	277.39	9.4	

In theory, compounds with very close values of pK_a should be separated when the electrolyte solution pH is nearby the difference pK_a–Log 2 [11]. According to this equation, the experiments were carried out at pH 9.5 using both a 56 mM phosphoric acid and a 56 mM boric acid electrolyte solution. In the first case, TADs were not completely separated, probably due to the ion pairing effect.

In the second case, the secondary amines can be separated, but the tertiary amines are only partially resolved (Fig. 2).

Because of the greater positive charge, the electrophoretic mobility of the tertiary amines is higher than the electrophoretic mobility of the secondary amines. Moreover, at this pH the eof is high and prevents the differences of mobility from getting larger.

A different choice could be:

- an acidic pH to lower the eof, whose influence on the mobility is negligible, but, on the other hand, in acidic environment, the TADs are fully positively charged, so that their charge-to-mass ratio and mobility are so close that it would be very difficult to have a separation.
- a permanent coated capillary, to strongly reduce the eof and the absorption on the capillary wall. Unfortunately, this kind of capillary is not stable at high pH.
- So the *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine (TMBD) Fig. 3 as dynamic coating was used.

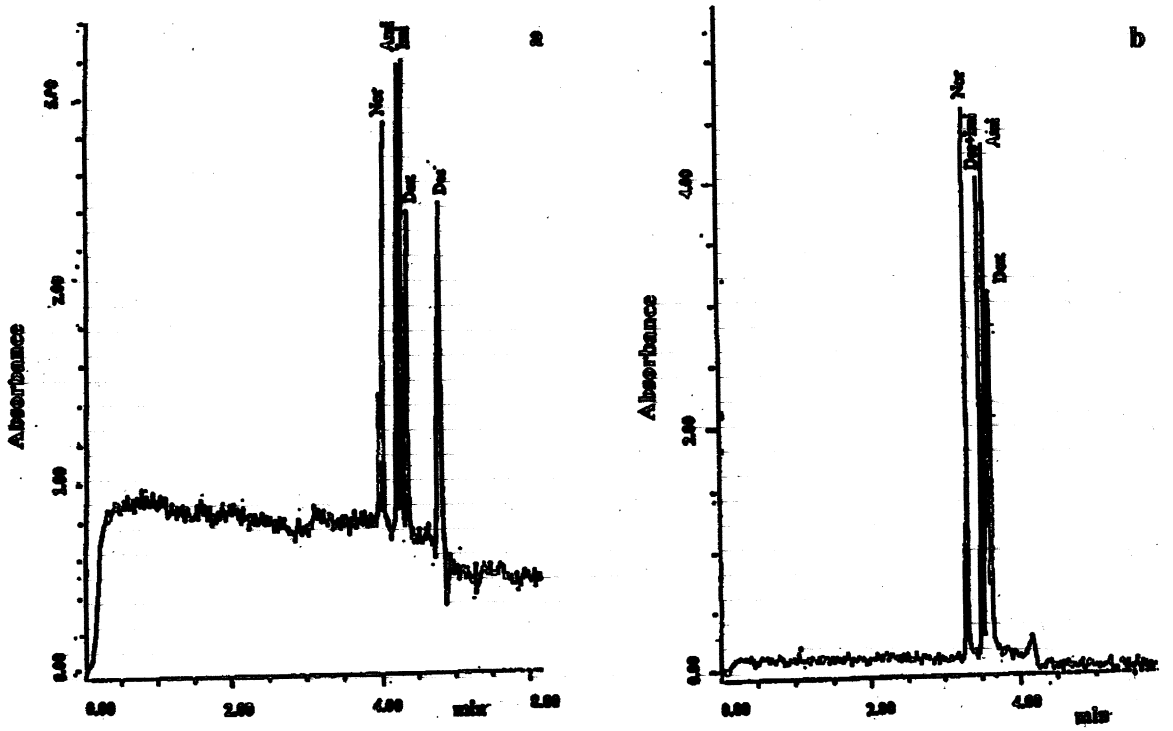

Fig. 2. Separation of TADs by using: (a) 56 mM boric acid pH 9.5; (b) 56 mM phosphoric acid pH 9.5.

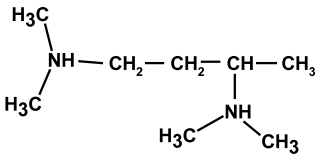

Fig. 3. Structure of *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine (TMBD).

Table 2
Chemical and physical properties of TMBD

Formula	$C_8H_{20}N_2$
Molecular mass	144.26
Boiling point (at 12 Torr)	55–56 °C
Relative density (a 20 °C)	0.802
UV transparency	up to 200 nm
Purity	>99% (GC)
pK_{a_1}	7.9
pK_{a_2}	10.3

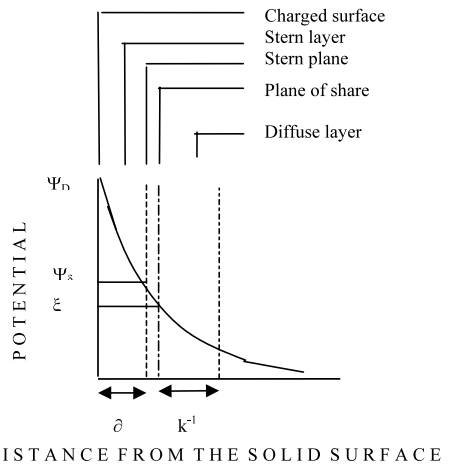

Fig. 4. Electrical potential as function of the distance from the solid surface.

This additive, already used for the separation of proteins [12], was added to the electrolyte solution, allowing the separation of TADs and obtaining a good selectivity and reproducibility.

TMBD is an aliphatic tertiary diamine whose chemical and physical properties are tabulated in Table 2. TMBD masks the negative charges due to the ionisation of the silanol groups on the capillary wall changing the zeta potential on the surface of the capillary and, therefore, the eof. In fact, the interactions between the tertiary amines of TMBD and silanol groups occur, so that TMBD molecules are strongly absorbed in the electric double-layer (Stern region) which is between the surface of the capillary and the electrolyte solution. [13,14]. A graphic representation of the progress of the electric potential in the electric double-layer is plotted in Fig. 4.

3.1. Electroosmotic flow (eof)

A neutral molecule, mesityl oxide, and a 40 mM TMBD solution at different pHs were used to test the eof (Fig. 5). As it can be seen, the eof is cathodic at pH 8 and anodic at lower pHs. At pH 8 the electroosmotic mobility is lower than the electroosmotic mobility measured with other electrolyte solutions [15]. The electrophoretic mobility was calculated by the following equation: $\mu_{eof} = (l \cdot L)/(V \cdot t_m)$, where l and L are the length to the detector and the total length of the capillary, respectively. V is the applied electrical potential and t_m is the migration time (s) of the mesityl oxide.

According to the pK_as reported in Table 2, TMBD is partially protonated at pH 8. Since TMBD is placed in the Stern region, the negative charge density due to the dissociation of the silanol groups is reduced. But as the eof is still cathodic, it means that the absorption of the TMBD is not enough to suppress the negative

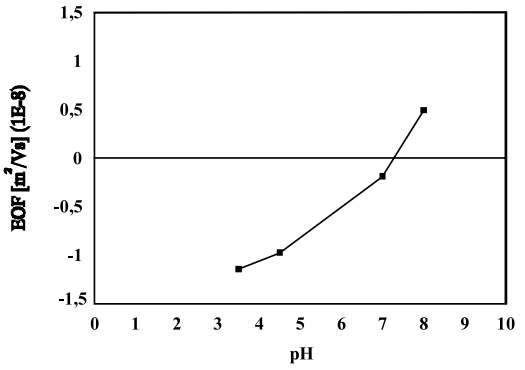

Fig. 5. Dependence of eof from 40 mM TMBD/FA electrolyte solution pH.

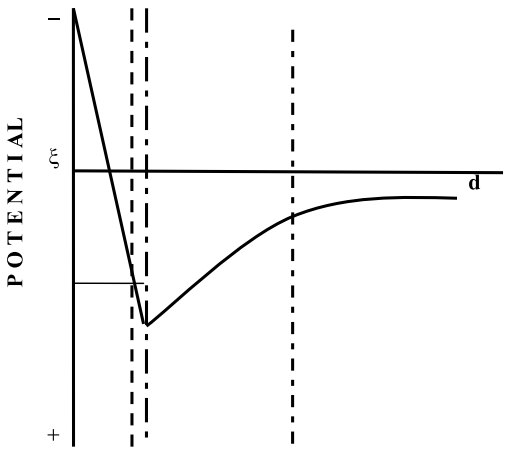

Fig. 6. Action of TMBD at the solid–liquid interface and effect on the eof.

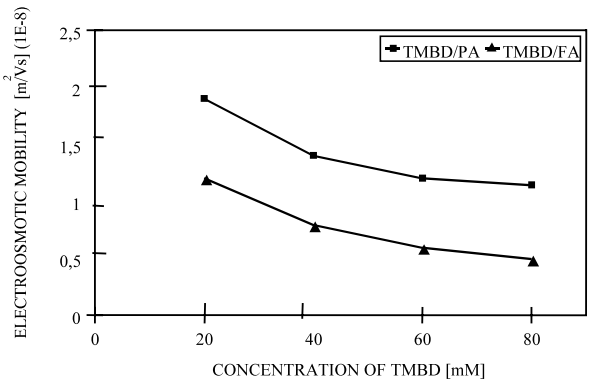

Fig. 7. Dependence of eof from the concentration of TMBD titrated with phosphoric acid of formic acid.

charges on the capillary wall, so that the zeta potential (ζ) is still negative but reduced. At lower pHs the positive charge density due to the TMBD is able to suppress the negative charges of silanol groups, so the zeta potential is reversed (from negative to positive). The TMBD behaviour in the Stern region, is not just due to the electrostatic interactions between the positive charges of the amine and the negative charges of the silanol groups, but also to non-electrostatic interactions between non-dissociated silanol groups and/or siloxane groups [16]. Lowering the pH, the negative charge density on the capillary wall decreases and the positive zeta potential increases because of the TMBD effect in the Stern region [17]. The effect of TMBD on the zeta potential can be described by the graphic representation in Fig. 6.

The effect of the concentration of TMBD solution on the electroosmotic mobility has been studied at pH 8. At this pH, which is close to the pK_{a_2}, the TMBD is a good buffer. For this reason it was possible to check the influence of the kind of acid used to adjust the pH of the solution of TMBD. For this purpose both TMBD/formic acid (TMBD/FA) and TMBD/phosphoric acid (TMBD/PA) solution at pH 8 and at different concentrations have been used (Fig. 7). Each series of measures (TMBD/FA or TMBD/PA) was run in quintuplicate in order to verify the repeatability of the values of the electroosmotic mobility.

The electroosmotic mobility is reduced when the concentration of TMBD is increased in both cases. The difference between the two acids employed for obtaining a pH 8 TMBD solution can be noted. The electroosmotic mobility values measured with a TMBD/FA solution are lower than those measured with a TMBD/PA solution. This could likely be due to a different nature of the formic acid (monoprotic) and the phosphoric acid (poliprotic). Nevertheless, the electroosmotic mobility shows a parallel course in both cases. This means that the same mechanism occurs and that it is responsible for the variation of the eof depending on the concentration of TMBD in both TMBD/FA and in TMBD/PA solutions. The variation of the eof, depending on the increasing concentration of aliphatic amines in the electrolyte solution, was already observed [13,14]. The same effect is what is likely to happen in this case, where the influence of the counter ion is shown. The change of the eof could also be due to the difference of the ionic strength of the two solutions (TMBD/FA and TMBD/PA) [18].

3.2. Action of TMBD

The masking action of TMBD on the silanol groups was investigated using different pH values of the TMBD solution in the separation of basic proteins: cytochrome c, lysozyme, ribonuclease A and α-chymotrypsinogen A (Table 3). An aqueous

solution of each protein was prepared and then stored at −20 °C. The separation of the tabulated proteins was carried out using 40 mM TMBD titrated with formic acid to a pH ranging from 3.5 to 8.0 (Fig. 8). The electropherograms show that the proteins are separated at acidic pHs, as expected, ever since the eof is anodic.

Table 3
Chemical properties of basic proteins used to investigate the action of TMBD

Protein	Acronym	Molecolar mass (Da)	Isoelectric point	Ref.
Cytochrome c	Cyt	12 400	9.5	A
Lysozyme	Lys	14 400	11.0	B
Ribonuclease A	RNase	13 700	9.5	B
α-Chymotrypsinogen A	Chy	25 000	9.6	A

A P.G. Righetti, T. Caravaggio, J. Chromatogr. 127 (1976) 1–28.B C.C. Worthington (Ed.), Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, 1988, pp. 219–299.

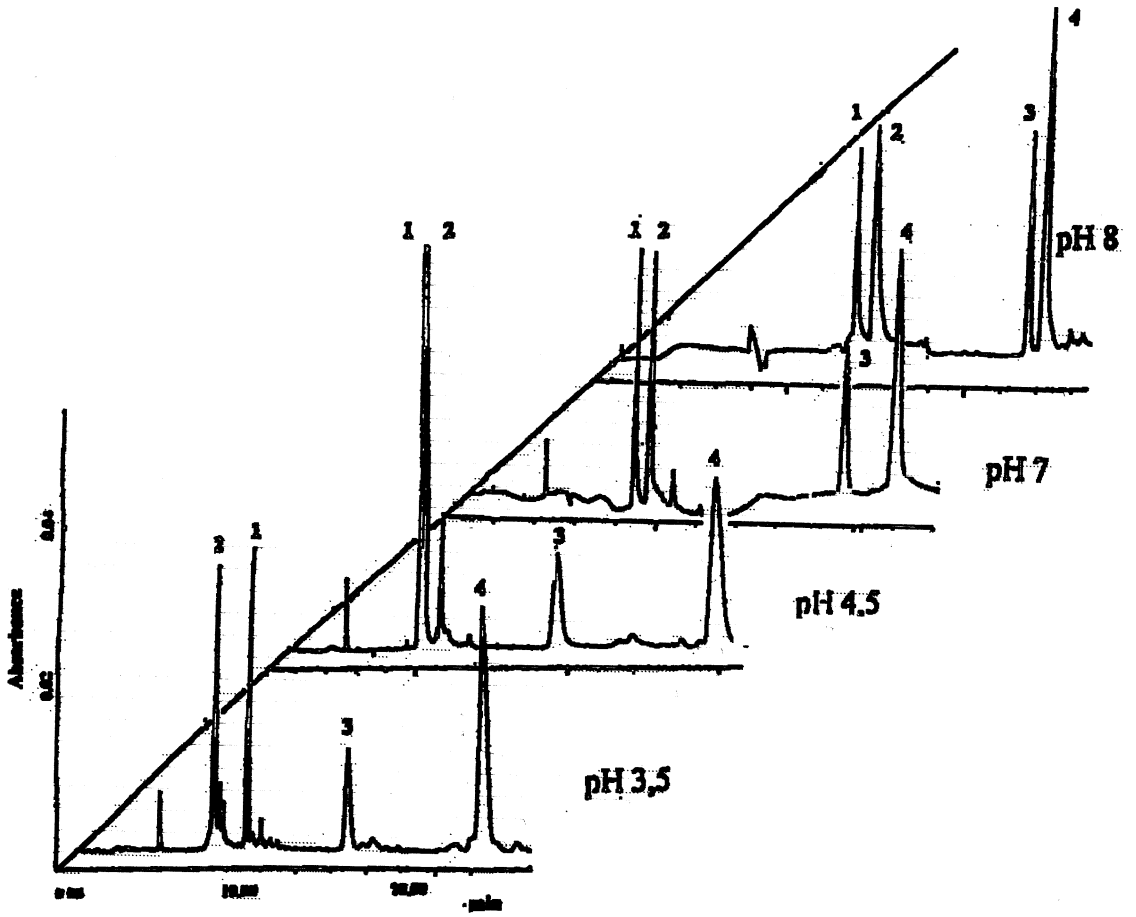

Fig. 8. Separation of cytochrome c, lysozome, ribonuclease A, α-chrymotrypsinogen A. Conditions: fused silica capillary (50 μm ID, 570 mm total length, 550 mm to the detector); 40 mM TMBD pH 3.5–8.0.

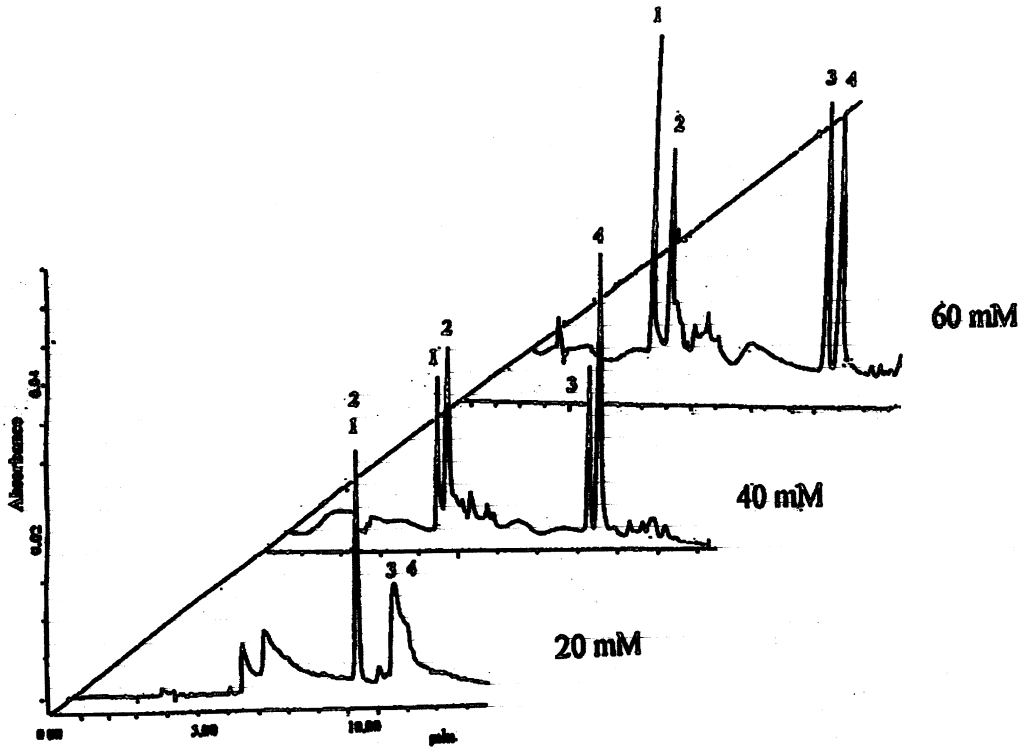

Fig. 9. Separation of cytochrome c, lysozome, ribonuclease A, α-chrymotrypsinogen A. Conditions: fused silica capillary (50 μm ID, 570 mm total length, 550 mm to the detector); 20–60 mM TMBD pH 8.

At pH 8, in spite of the cathodic eof, the proteins are however well separated because of the TMBD presence. Fixing the pH at 8.0, the investigation was carried out with different TMBD concentrations ranging from 20 to 60 mM (Fig. 9). The resolution is poor with 20 mM TMBD and it is clear, by the peak tailing and broadening, the interaction of proteins on the capillary wall. Increasing the concentration of TMBD the resolution is improved.

Also the influence of TMBD concentration on the apparent mobility of TADs was investigated. The experiment was carried out at pH 9.5 using 40, 60, 100 and 150 mM TMBD (Fig. 10). An appropriate quantity of concentrated phosphoric acid (85% p/v, $d = 1.70$ g ml^{-1}, MW = 98) was added to an aqueous solution of TMBD (99% p/v, $d = 0.802$ g l^{-1}, MW = 144.26), obtaining 40 or 60 or 100 or 150 mM solution and 56 mM H_3PO_4 solution pH 9.5. TADs, as hydrochloride

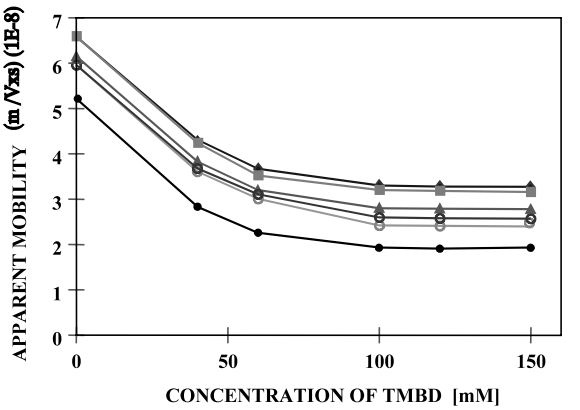

Fig. 10. Apparent mobility of ○ amitriptyline, ■ nortriptyline; ◆ desipramyne; ○ doxepin; ▲ imipramine; ● mesityl oxide as function of the concentration of TMBD. Conditions: fused silica capillary (50 μm ID, 570 mm total length, 550 mm to the detector); 40, 60, 100, 150 mM TMBD pH 9.5.

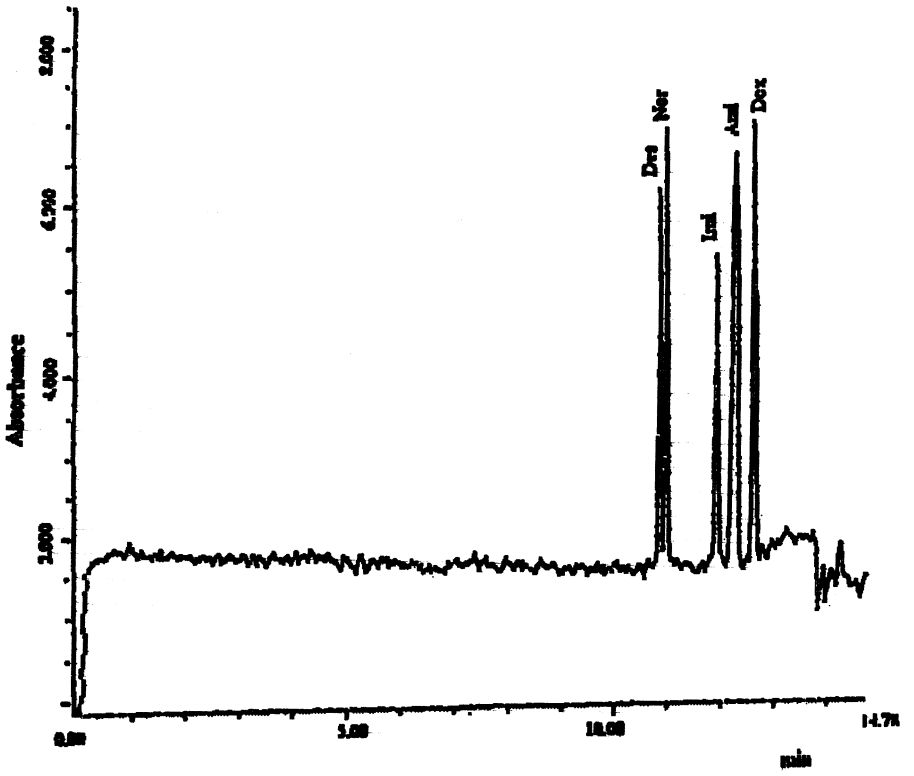

Fig. 11. Separation of TADs using 100 mM TMBD pH 9.5.

salts, were well solubilised in water and no precipitation into the capillary occurred. The samples were analysed by CE at the following concentrations: nortriptyline and desipramyne 0.02 mg ml^{-1}, amitriptyline and doxepin 0.03 mg ml^{-1}, imipramine 0.04 mg ml^{-1}. They were run in triplicate and showed a good stability, reproducibility of both the migration time and the area values. A solution of mesityl oxide was used to measure the electroosmotic mobility which decreases when the TMBD concentration is increased up to a concentration value of 100 mM. After that, the mobility becomes constant. In the same way, the apparent mobility of each TAD decreases till 100 mM, coming constant at higher concentrations of TMBD. This is the consequence of the decreasing of the electroosmotic mobility. The zero point of graphic in Fig. 10 related to the absence of TMBD, was measured by using a 56 mM phosphoric acid solution which is the same concentration used to titrate the TMBD solutions. Besides, at 100 mM TMBD, TADs are completely separated (Fig. 11).

The concentration of 100 mM corresponds to a minimum value of eof which allows the resolution of TADs as shown in Fig. 10. The masking action of TMBD can be seen in limiting the interaction between the silanol groups and the amino groups of TADs. To evaluate the influence of the composition of the electrolyte solution on the electrophoretic mobility of the analytes, the triphenyl ammonium iodide, a quaternary ammonium salt, was added to the sample solutions. Its positive charge is not affected by the change of the pH of the electrolyte solution. Moreover, because no interactions occur between the triphenyl ammonium iodide and the electrolyte solution, its electrophoretic mobility is constant in a wide range of pH [19].

Efficiency, as well, depends on the TMBD concentration (Fig. 12). The number of the theoretical plates increases adding the TMBD in the electrolyte solution. This increment is greater for the two more basic TADs, desipramyne and nortriptyline. It is also interesting to note that the minimum value (100 mM) of TMBD corresponds to the maximum masking of the silanol groups and so the maximum value of the number of the theoretical plates. The selectivity is also improved. Fig. 13 shows the relation of the selectivity coefficient versus the concentration of TMBD. The selectivity coefficient was calculated by the equation: $r_{ij} = \mu_i/\mu_j$ where μ_i is related to the compound with the highest mobility.

4. Conclusion

The optimal conditions for separating the five TADs seem to be a 100 mM TMBD electrolyte solution at pH 9.5. Electrolyte solutions made of TMBD and a polyprotic acid allow to check the eof and limit the interactions of basic compounds on the capillary wall even at a high pH. The complete resolution of TADs was achieved by reducing the eof. Consequently, the efficiency and the selectivity were improved.

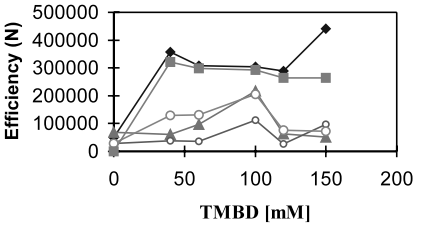

Fig. 12. Efficiency of TADs vs. the TMBD concentration: ○ amitriptyline, ■ nortriptyline; ◆ desipramyne; ○ doxepin; ▲ imipramine.

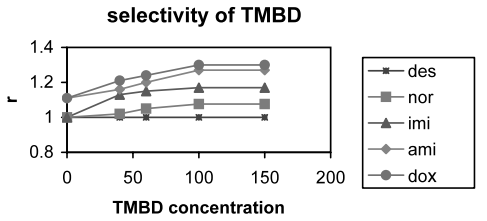

Fig. 13. Selectivity coefficient vs. different concentrations of TMBD.

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