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Optimization of the separation of β-agonists by capillary electrophoresis on untreated and C_{18} bonded silica capillaries

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Abstract

The conditions of the separation of ten β-agonists by capillary zone electrophoresis were studied. Several buffers were tested at different ionic strengths and different pH values. The experiments were carried out on two different supports, i.e. an untreated fused-silica capillary and a C_{18} covalently bonded silica capillary. The results showed that the optimum pH value was the same for the two capillaries. Separation efficiencies were slightly better for the fused-silica capillary whereas better selectivity and repeatability were obtained with the C_{18} bonded capillary, under optimal conditions.

1. Introduction

Beta-adrenergic agonists, commonly named β-agonists, are used as bronchodilators in human and veterinary therapeutics for the treatment of pulmonary diseases. When used as animal feed additives, β-agonists can also act as repartitioning agents, by increasing protein accretion and decreasing the lipogenesis. Thus, they cause a shift in carcass composition, improving the yield of the farm animals [1].

β-Agonists are considered anabolic substances within the European Community and their use as animal feed additives is prohibited. A number of β-agonists are however known to be illegally used, thus imposing serious food safety problems. Consequently, accurate multi-compound analyses are required for effective control.

β-Agonists are mainly phenylethanolamines, variously substituted on their aryl moiety and terminal amino group. In simple chemical terms, the chemical structure of this diverse group of drugs can be defined as arylhydroxyalkylamines possessing a common functionality: the presence of a β-hydroxyamino group on the side-chain.

Although not known accurately, the pK_a values of the β-agonists, are assumed to be in the range 7–9 [2].

Several analytical techniques have already been used for the analysis of these polar and ionic compounds, including GC–MS [3–5] and HPLC with UV [6], fluorescence [7], or MS detection [8,9]. Several authors have also demonstrated the use of capillary electrophoresis for their analysis in pharmaceutical formulations [10], determination of drug related impurities [11] and chiral analysis of enantiomeric forms of β-agonists [12,13]. Recently, four β-agonists have been analysed in calf urine using on-line

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isotachophoresis–capillary zone electrophoresis (ITP–CZE) prior to mass spectrometric detection [2].

Capillary zone electrophoresis (CZE) is a very efficient separation technique with a high resolution power, suitable for the separation of ionic compounds even with very small differences in their electrophoretic mobilities. The separation mechanism is based on differences in solute size and charge at a given pH.

In a non-coated fused-silica capillary, the interface between the silanol groups of the tube wall and the electrophoretic buffer consists of a double-layer which tends to make the flow of liquid migrate towards the cathode. This bulk movement of liquid is called the electroosmotic flow (EOF). In CZE, the control of this EOF is very important for improving the separation and shortening the analysis time. It can be adjusted by changing the nature of the buffer, its viscosity (temperature), its ionic strength, or by controlling the voltage of the separation. The most commonly used optimization techniques in CZE are changing the pH of the running buffer to change the charge/mass ratio of the ions, using additives to decrease the EOF, or the use of coated columns.

Some of the problems encountered when using a non-coated fused-silica capillary are the possible variations in the EOF and the adsorption of charged molecules on the capillary surface. Thus rinsing of unmodified capillaries is an important factor in obtaining stable results.

Coated capillary columns are employed to reduce the EOF, to control the migration time and to improve column efficiency by minimizing solute adsorption on the bare silica wall. Among the different coated columns, the highly hydrophobic C_{18} bonded phase–well-known in HPLC–attracted attention because of its stability over time at neutral pH and its ability to reduce EOF and decrease solute–wall interactions [14].

In this paper, after preliminary experiments, we have studied the separation of ten β-agonists by CZE on two supports (uncoated fused-silica and C_{18} bonded phase). The structures of these β-agonists are shown in Fig. 1. The influence of the pH of running buffer has been examined for both columns. The separation efficiency of the two systems is evaluated, and their repeatability

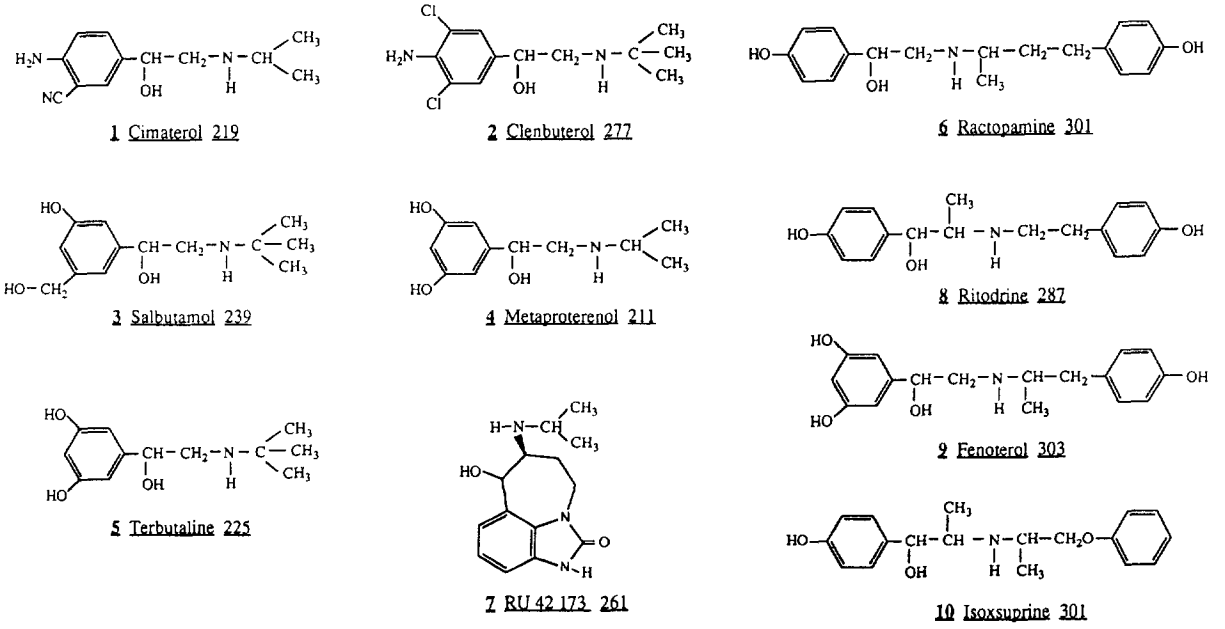

Fig. 1. Structures of the ten β-agonists studied.

is discussed in terms of migration times, relative migration times, separation efficiencies, and normalized peak areas.

2. Experimental

2.1. Apparatus

Electrophoresis was carried out on a Hewlett-Packard HP ^{3D}CE system (Hewlett-Packard, Wilmington, DE, USA) with a built-in UV diode-array detector and DOS Windows-type, data-analysis software.

Separations were performed using two different columns. A non-coated fused-silica capillary (68.5 cm × 50 μm I.D., 60 cm effective length) (Hewlett-Packard) and a C_{18} bonded phase capillary column (CElect-H250) (68.5 cm × 50 μm I.D., 60 cm effective length) (Supelco, Bellefonte, PA, USA).

All experiments were carried out in the cationic mode, applying a voltage of 15–30 kV. Hydrostatic injection was applied for 2–6 s at 50 mbar, followed by a 4-s flush with buffer. Detection was performed at a wavelength of 200 nm. The temperature was kept constant at 25°C.

Before its use, a new fused-silica capillary was conditioned with 1 *M* NaOH for 20 min at 40°C, then with 0.1 *M* NaOH for 10 min at 40°C, rinsed with water for 20 min at 25°C and finally with the running buffer. In order to improve the migration-time and peak-shape reproducibilities, after each run the system was programmed for the following successive operations: 5 min rinsing with 0.1 *M* NaOH followed by 5 min with running buffer.

The C_{18} bonded phase capillary was conditioned for 1 min with 0.1 *M* NaOH, for 5 min with water and for 5 min with running buffer, repeating the wash/rinse cycle over a 2-h period. After each run the column was rinsed for 5 min with the running buffer, and every ten runs, washed for 1 min with 0.1 *M* NaOH, and for 5 min with the running buffer.

The running buffers used in these experiments were prepared with tris(hydroxymethyl)aminomethane (Tris) (Sigma, La Verpillière, France) in deionized water (Milli Q system, Millipore, Bedford, MA, USA) and adjusted at pH 5–9 with acetic acid. All buffer solutions were filtered through 0.45-μm membrane filters (Millipore, Molsheim, France) before use.

2.2. Samples

Salbutamol, fenoterol hydrobromide, metaproterenol hemisulfate salt, ritodrine hydrochloride, isoxsuprine hydrochloride, and terbutaline hemisulfate salt were purchased from Sigma. Cimaterol and clenbuterol hydrochloride were obtained from Boehringer (Ingelheim, Germany) and ractopamine hydrochloride from Eli Lilly (St. Cloud, France). RU 42 173 was kindly provided by Roussel Uclaf (Romainville, France).

Standard solutions were prepared in deionized water at a concentration of 100 ng μl^{-1}. A working solution was prepared by mixing 100 μl of the ten standard solutions, which resulted in a concentration of 10 ng μl^{-1} for each compound.

Each β-agonist was identified in the mixture by automated spiking achieved by co-injection of each individual component and the mixture under the same conditions as described by McLaughlin et al. [15] and Altria and Luscombe [16].

3. Results and discussion

3.1. Preliminary experiments

The optimization of the β-agonists separation was performed on the fused-silica capillary. The first parameters studied were the nature and the ionic strength of the running buffer, and the applied voltage.

For preliminary experiments, the applied voltage was set at 15 kV, and the following buffer solutions (20 m*M*) were tested: sodium citrate (pH 5), acetate (pH 5), phosphate (pH 7), borate (pH 9) and finally Tris (pH 5).

None of the first four buffer systems gave satisfying results. Indeed, when sodium citrate

was used, a high current was produced and baseline instability was observed. With acetate, peak shape distortions were obtained, which should be explained by differences in conductivity between the sample and the buffer [17]. Phosphate gave no satisfactory separation and borate gave no separation at all of the compounds studied. Ackermans et al. [10] obtained some satisfactory results with Tris (pH 5) for the determination of some β-agonists, and this buffer system was selected for further optimization.

The influence of the ionic strength of the Tris buffer was then studied at pH 5. The use of Tris at 50 m*M* instead of 20 m*M* resulted in improvement of the resolution without increasing too much the current intensity. A 100 m*M* Tris solution increased the migration times but did not improve the separation. Thus, the Tris buffer concentration was fixed at 50 m*M*.

An increase of the applied voltage from 15 kV to 30 kV neither improved the separation, nor changed the elution order. It did, however, reduce the migration times of the β-agonists. A similar trend was observed by Lukkari et al. [18] in a study using micellar electrokinetic capillary chromatography (MEKC) for the separation of nine β-blockers. From these observations, 30 kV was chosen as the working voltage.

3.2. Influence of buffer pH

The influence of buffer pH on the migration times of the β-agonists was investigated with a buffer concentration of 50 m*M* Tris and an applied voltage of 30 kV. The pH was changed from 5 to 9 and two different capillaries were compared: an uncoated fused-silica capillary and a C_{18} bonded capillary.

The plots of the migration times of each β-agonist studied versus pH are given in Figs. 2a and b, for the uncoated and C_{18} bonded capillaries, respectively. It appears that, for a given pH, migration times are longer for the C_{18} bonded capillary than for the untreated one. This can be attributed to the well-known decrease of the EOF on bonded capillaries [14].

Moreover it can be seen that for a given capillary, the shapes of the plots are very similar from one compound to another, indicating that each β-agonist shows the same behaviour on changing the buffer pH. Little difference is observed between the two types of capillaries. Indeed, the migration times of all compounds decrease with increasing pH. This is in agreement with an increase of the electroosmotic mobilities with pH [17,19]. Nevertheless, some differences between the two capillaries can be pointed out, especially for the pH range 8–9.

For the uncoated fused-silica capillary, the migration times did not change from pH 5 to pH 6, and then decreased uniformly from pH 6 to pH 9. For the C_{18} bonded capillary, the migration times decreased gradually with an increase of the pH from 5 to 8. A minimum was observed at pH 8, and then the migration times increased with a further increase of the pH to 8.3. At a pH above 8.6, the migration times decreased again, whereas some little discrepancy could be observed for some of the β-agonists in the pH range 8.3–8.6.

This particular behaviour of the β-agonists on the C_{18} bonded capillary between pH 8 and 9 remains unexplained. In a study on the separation of metallothioneins on an untreated fused-silica capillary, Liu et al. [20] obtained an incurvated graph, which was interpreted to be a consequence of the variations in the ionic strength of the solutions due to the buffer pH titration. In our case, with Tris buffer a decrease in pH corresponds to an increase of the number of ions, and hence an increase in the ionic strength, which results in an increased migration time, as described by Vindevogel and Sandra [21]. Thus, it seems that the migration-time variations between pH 8 and pH 8.6 observed on the C_{18} capillary are not related to the titration of the Tris buffer.

The increased migration times observed on the C_{18} bonded capillary at pH 8–8.6 could be tentatively interpreted, considering that for these pH values close to the pK_a values of β-agonists, some solute partitioning should occur between the running buffer and the hydrophobic octadecyl groups bonded to the capillary wall. Thus, some retention should participate in the separation process, resulting in a kind of electrically

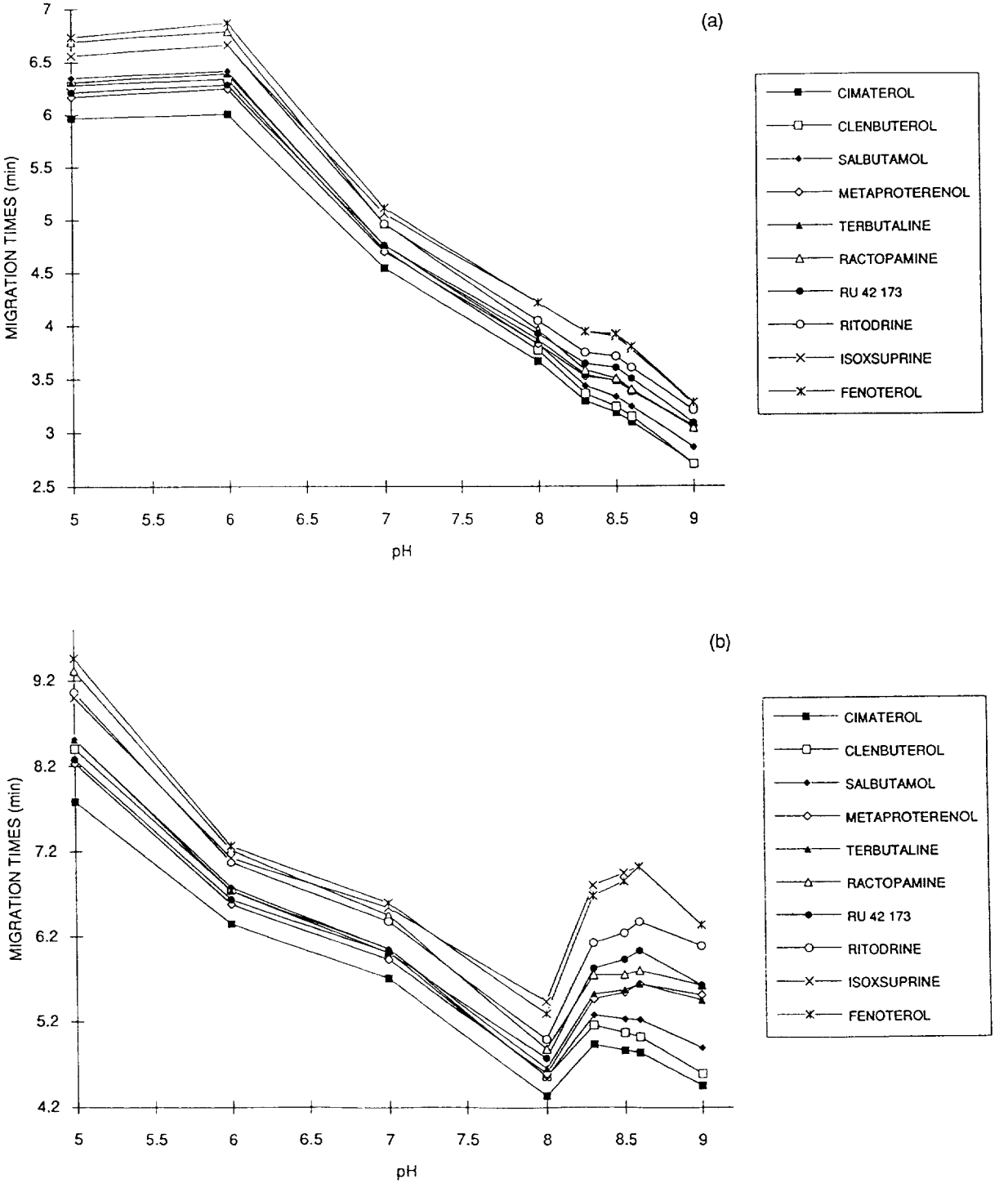

Fig. 2. Plot of migration times of the β-agonists versus pH obtained (a) on fused-silica capillary and (b) on C_{18} bonded capillary. Conditions: running buffer, Tris 50 mM; voltage, 30 kV; temperature, 25°C; UV detection at 200 nm.

driven open-tubular liquid chromatography as described by Bruin et al. [22].

The elution orders of the β-agonists at pH 5 to pH 9 are shown in Figs. 3a and b, for the uncoated and C_{18} bonded capillaries respectively, according to a scheme proposed by Lukkari et al. [23]. These orders are identical for the two capillaries for a given pH, although no complete separation could be obtained on the silica capillary. On the other hand, a small change in pH can change the elution order, except for cimaterol which is always eluted first, irrespective of the capillary employed. This effect could result in some limitation concerning the robustness of the separation.

No clear correlation between the migration times and the structure of the β-agonists could be deduced from our experiments, since numerous factors–such as the tendency of some β-agonists to interact with the capillary wall and their ability to form doubly-charged species–prevent precise predictions. Nevertheless, at pH 5, at which one can consider all β-agonists positively charged, the elution order is very close to the order of increasing molecular masses of the compounds. Exceptions are for cimaterol, clenbuterol and RU 42 173 (Fig. 1) which bear two amino groups and thus can be eluted faster.

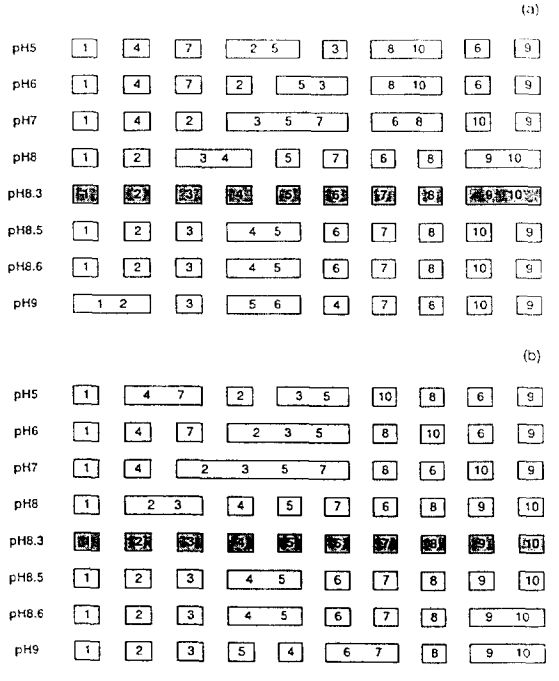

Fig. 3. Elution order of the ten β-agonists from pH 5 to pH 9 obtained (a) on fused-silica capillary and (b) on C_{18} bonded capillary. Conditions: running buffer, Tris 50 m*M*; voltage, 30 kV; temperature, 25°C; UV detection at 200 nm. (for compound numbers see Fig. 1).

Finally, complete separation of the mixture of β-agonists can be achieved on the C_{18} bonded capillary within 6.8 min at pH 8.3. For the untreated fused-silica capillary the optimum pH is also set at 8.3, which allows the separation of nine of the ten β-agonists studied, within 4 min. The electropherograms obtained under these conditions are shown in Figs. 4a and b for the two types of capillaries.

3.3. Repeatability

The repeatability of the separations was assessed for the two capillaries and at different pH values. Repeatabilities were estimated from the means and relative standard deviations (R.S.D.), for migration times (MT) and for relative migration times (RMT) calculated relatively to cimaterol:

$$RMT = MT/MT_c$$

where MT is the migration time of the actual β-agonist, and MT_c is the migration time of cimaterol, for which the RMT is unity. Selected results are shown in Table 1.

The R.S.D. ($n = 10$) of MT values varied from 0.30 to 1.62% for untreated silica and from 0.10 to 2.83% for C_{18} bonded capillary. For RMT, values ranging from 0.07 to 0.20% and from 0.07 to 0.50% were obtained for the untreated and bonded capillaries, respectively. These values show that highly repeatable separations were obtained on the C_{18} bonded support as well as on the silica capillary. Moreover, it should be noted that the stability of the MT is better on the bonded capillary for pH values near the optimum value (see bold values in Table 1).

The migration-time drift described in the literature [24] for C_{18} bonded capillaries, was not observed in this study, especially for pH values close to the optimum pH (8.3). A slight drift was

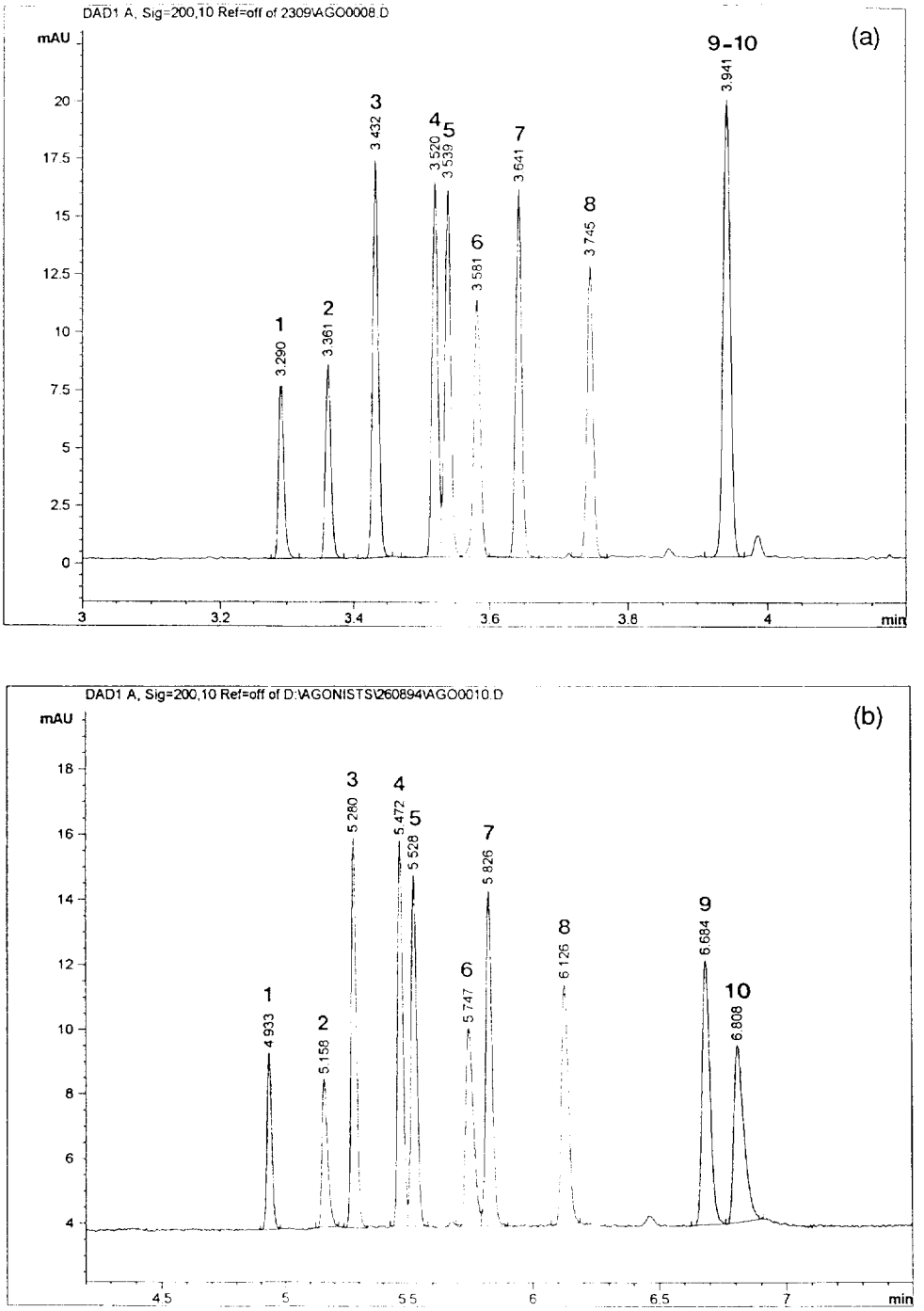

Fig. 4. Electropherograms of the β-agonists mixture obtained (a) on fused-silica capillary and (b) on C_{18} bonded capillary. Conditions: running buffer, Tris 50 mM (pH 8.3); voltage, 30 kV; temperature, 25°C; UV detection at 200 nm.

Table 1
Selected results of relative standard deviations (R.S.D.) of migration times (MT) and relative migration times (RMT)

		Metaproterenol			Terbutaline			RU 42173		
		pH 5	pH 7	pH 8.3	pH 5	pH 7	pH 8.3	pH 5	pH 7	pH 8.3
R.S.D. (%)	MT silica	0.41	0.33	1.45	0.41	0.32	1.49	0.42	0.32	1.52
	MT C_{18}	2.5	2.08	**0.15**	2.61	2.13	**0.14**	2.52	2.07	**0.17**
R.S.D. (%)	RMT silica	0.07	0.07	0.1	0.18	0.09	0.11	0.08	0.09	0.14
	RMT C_{18}	0.18	0.13	**0.07**	0.25	0.19	**0.07**	0.26	0.13	**0.09**

nevertheless observed at pH 5, which is the cause of the higher R.S.D. values obtained for RMT at this pH (Table 1).

The performance stability of the two systems was also assessed from the separation efficiency, measured by the number of theoretical plates according to the formula:

$$N = 5.54(\mathrm{MT}/w_{h1/2})^2$$

where N is the number of theoretical plates and $w_{h1/2}$ is the peak width at half-height.

The results indicated that for the two capillaries, best efficiencies were obtained at pH 8.3. At this pH, efficiencies vary between 200 000 and 500 000 theoretical plates, which corresponds to approximately 350 000–800 000 theoretical plates per meter of capillary. As a general rule, slightly better efficiencies are obtained with the untreated silica capillary, irrespective of the pH selected. However, especially at the optimum pH, a constant decrease in efficiency was observed from injection to injection for the silica capillary (Fig. 5). This phenomenon was not

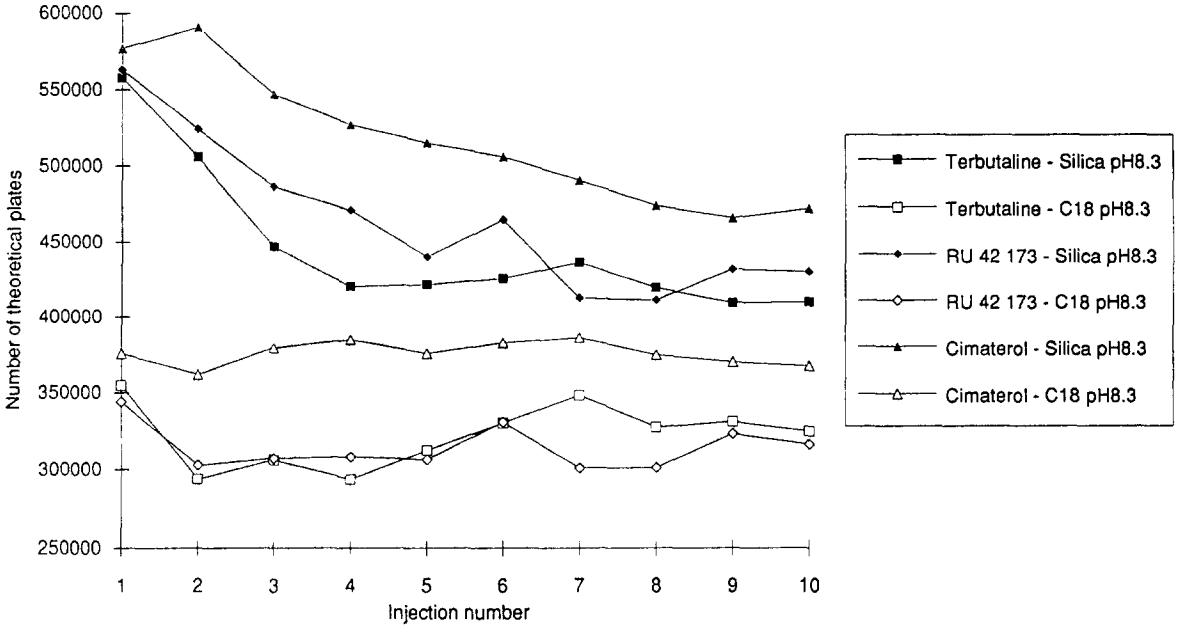

Fig. 5. Number of theoretical plate versus injection number on fused-silica capillary (filled symbols) and on C_{18} bonded capillary (open symbols).

observed for the bonded capillary, which showed a stable performance over time (Fig. 5), resulting in better R.S.D. values of the efficiency.

Adsorption phenomena on fused-silica capillary walls have been extensively described [17,24], and they seem to occur not only for proteins, but also for small ionic molecules like β-agonists, in spite of frequent sodium hydroxide rinsing. In the case of the C_{18} bonded capillary, adsorption is much less important and performance stability seems better, and sodium hydroxide rinsing between runs is unnecessary.

To evaluate the suitability of the method for quantitative determinations, we measured the peak areas of each β-agonist. Normalized peak areas (NPAs) (i.e. peak areas divided by migration times [15,25]) were used in mean and R.S.D. calculations ($n = 9$).

Table 2 shows the mean and R.S.D. values of NPAs calculated for cimaterol, RU 42 173 and ractopamine at two different pH values. Results show that NPA values obtained on the fused-silica capillary are always lower than on the C_{18} bonded capillary for all β-agonist studied. This observation is quite surprising since the two columns have identical internal diameters (50 μm) as well as external diameters (360 and 363 μm, respectively). It may, however, be consistent with an adsorption phenomenon as revealed by the previously mentioned need to frequently rinse the silica capillary. Moreover, adsorption would be associated with lower theoretical plate numbers; work is in progress to tentatively explain this discrepancy.

The R.S.D. values vary in the same range (i.e. between 0.5 and 2.5%, depending on the compound considered) for the two pH values and the two capillaries. Although HPLC has been mentioned to give even better results [10], these values indicate a good precision of the method for quantitative purposes, taking into account the low concentration test mixture employed in this study (10 $\mu g\,ml^{-1}$). The use of higher sample concentrations would give better R.S.D. values since in CE it is well known that peak area precision improves with increased sample concentration [26,27].

4. Conclusion

Good separation of the β-agonists studied was obtained with both capillary systems under the conditions applied. The ten β-agonists studied can be separated with high efficiency in less than 7 min, with good repeatability. The ten peaks are baseline resolved in a migration window of less than 120 s using a C_{18} bonded silica capillary.

For separation of β-agonists, capillary electrophoresis was found to give significantly higher efficiency and speed compared to HPLC which requires the use of an ion-pairing agent [6–8]. The present CZE method offers certain advantages, including simple background electrolyte preparation, and does not require the long preconditioning and stabilization periods which are needed in ion-pairing HPLC.

Table 2
Selected results of means and R.S.D. of normalized peak areas (NPA) on fused and C_{18} bonded silica capillaries

pH		NPA-silica		NPA-C_{18}	
		Means	R.S.D. (%)	Means	R.S.D. (%)
5	Cimaterol	1.34	1.81	1.61	0.9
	RU 42 173	2.74	2.07	3.31	0.78
	Ractopamine	2.51	0.56	2.58	1.29
8.3	Cimaterol	1.34	1.42	1.45	1.67
	RU 42 173	2.82	1.95	3.04	1.87
	Ractopamine	2.12	1.54	2.24	1.13

The use of the C_{18} bonded capillary seems advantageous compared to the untreated capillary taking into account its greater stability, which results in a greater reproducibility with time. However, some potential drawbacks, such as batch-to-batch variability or coating stability, should be considered for this type of capillary which is still not widely used. Rinsing between runs was found to be necessary for the untreated fused-silica capillary, in order to bring the system back to the initial conditions, to keep the current constant from run to run, and to reduce adsorption on the capillary wall. Also the fact that no between-run rinsing is needed makes the C_{18} bonded capillary easier to use.

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