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Optimization of capillary electrophoretic separation of several inhibitors of the angiotensin-converting enzyme

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Abstract

Capillary electrophoretic separation of eight inhibitors of the angiotensin-converting enzyme, viz., enalapril, lisinopril, quinapril, fosinopril, perindopril, ramipril, benazepril and cilazapril, was investigated with respect to the following parameters: pH of the running buffer, organic modifiers and surfactants. The most critical parameter is the pH of the running buffer. The addition of sodium dodecyl sulfate had a negative influence on the peak symmetry, and selectivity was not improved. The separation of the eight compounds can be performed by means of two phosphate buffers (each 100 m*M*) at pH 7.0 and pH 6.25, respectively. This combination is necessary for the selective identification of structurally related substances because of their similar pK_a values.

Keywords: Capillary electrophoresis; Angiotensin-converting enzyme inhibitors

1. Introduction

The inhibitors of the angiotensin-converting enzyme (ACE) are widely used for the treatment of mild to moderate hypertension and heart failure, either alone or in conjunction with other drugs [1]. The first ACE inhibitor to be developed was captopril, a thiol-containing compound. However, because captopril caused some side effects and researchers believed that the thiol group was responsible for these effects, the next step was the development of non-thiol-containing ACE inhibitors [2].

There are three classes of these new ACE inhibitors, according to the particular moiety that enhances their binding to the zinc ion of the angiotensin-converting enzyme. The first class has a second ionizable carboxyl group; lisinopril is the only representative drug in this class, differing from the other inhibitors because it is not a prodrug. Fosinopril, a phosphorus-containing ACE inhibitor, belongs to the second class. Fosinopril is inactive but serves as a prodrug, being completely hydrolysed to the active diacid, fosinoprilate. Agents in the third class comprise all remaining ACE inhibitors, viz., enalapril maleate, quinapril, perindopril, ramipril, benazepril and cilazapril. These inhibitors possess an additional carboxylic acid ethyl ester and have the common property of acting as prodrugs, being converted to the active diacid by liver metabolism and intestinal enzymes [1,2].

Until now, high-performance liquid chromatography has been the major technique used for the determination of ACE inhibitors [3–18]. The same technique was also applied to collect the data in the European Pharmacopoeia monograph on enalapril maleate, lisinopril dihydrate and ramipril [19]. Capil-

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lary electrophoresis (CE) offers an alternative technique; however, analysis by means of CE has only been achieved so far for enalapril maleate, lisinopril and fosinopril. These studies have been limited to the determination and rotamer separation of enalapril maleate [20–22] and lisinopril [23]. One study has reported on the determination of fosinopril and its related impurities [24]. The aim of the present study was therefore to develop a selective method capable of separating a large number of structurally related ACE inhibitors by CE. The chemical structures of these inhibitors are represented in Fig. 1.

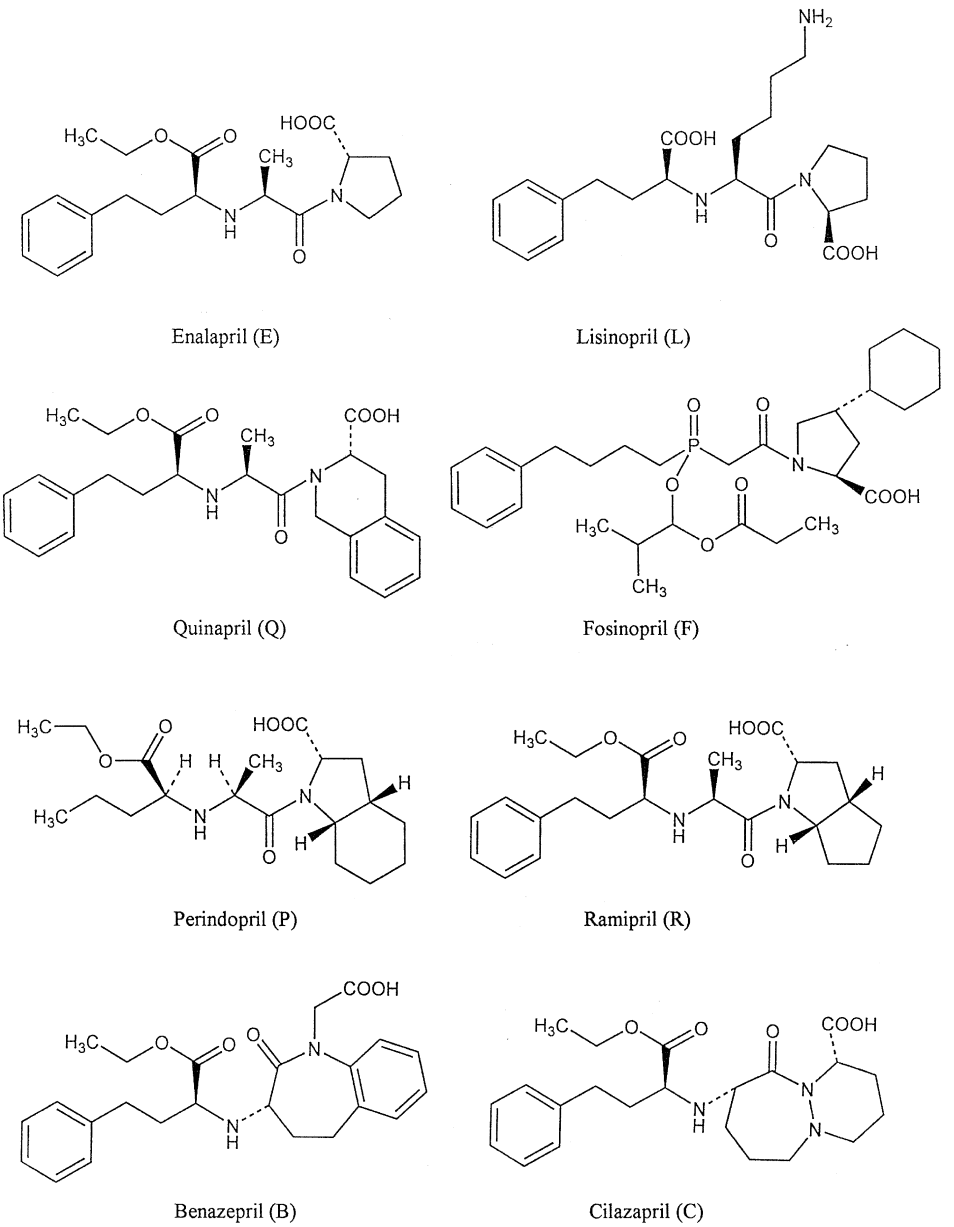

Fig. 1. Chemical structures of the ACE inhibitors.

2. Experimental

2.1. Instrumentation and electrophoresis procedure

The method was developed, and subsequent experiments were performed, on a Waters Quanta 4000 CE instrument (Millipore, Waters). The capillary used was a fused-silica capillary (either 60 cm or 93 cm in total length, with a length to the detector of 52.5 cm or 85.5 cm, respectively) and an internal diameter (I.D.) of 50 μm.

Hydrostatic injections were performed by lifting the sample vial approximately 10 cm above the height of the buffer vial for 10 s. For detection, the absorbance was measured by means of an on-line fixed-wavelength UV detector with a zinc discharge lamp and a 214-nm filter. The experiments were performed at 20 kV to 30 kV at room temperature (20±2°C). Data were collected on a Hewlett-Packard Integrator (HP 3396, Series II), which was also used for calculating the areas under the peaks.

2.2. Reagents

Sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate dihydrate (both analytical-reagent grade) were obtained from E. Merck (Germany). Enalapril maleate and lisinopril were purchased from Sigma (St. Louis, MO, USA). Quinapril was obtained from Parke-Davis, fosinopril from Bristol-Myers Squibb, perindopril from Servier, and benazepril from Ciba-Geigy.

Reference solutions for qualitative analysis of fosinopril, perindopril, ramipril, benazepril and cilazapril were prepared from the commercially available drugs (Fosinil, Coversyl, Tritace, Cibacen and Inhibace), by mixing the powder with the corresponding phosphate buffer. The suspensions were filtered through a membrane (0.45 μm).

2.3. Running buffers

During the development of the method, sodium phosphate buffers of different pH values were tested as running buffers. In the pH range of 2.0 to 4.5, a mixture of a phosphoric acid solution and sodium dihydrogenphosphate solution was used while in the pH range of 4.5 to 8.5, a mixture of a sodium dihydrogenphosphate solution and a disodium hydrogenphosphate solution was used.

Two sodium phosphate buffers were finally used as running buffers for the identification.

The first sodium phosphate buffer (pH 7.0; 100 m*M*) was prepared by adjusting the pH of a 100 m*M* disodium hydrogenphosphate solution to pH 7.0 by the addition of 100 m*M* sodium dihydrogenphosphate solution. The second sodium phosphate buffer (pH 6.25; 100 m*M*) was prepared by adjusting the pH of 100 m*M* sodium dihydrogenphosphate solution to pH 6.25 by addition of 100 m*M* disodium hydrogenphosphate solution.

2.4. Reference solutions

Reference solutions of the eight compounds were prepared at 100 μg ml^{-1} in the corresponding running buffer.

All samples and buffers were filtered through a Millipore 0.45-μm filter unit.

3. Results and discussion

3.1. Capillary zone electrophoresis

3.1.1. Influence of pH

Owing to the amphoteric character of ACE inhibitors, their retention is greatly influenced by pH (Fig. 2). Therefore, the principal option for modifying the separation method is to vary the pH. All ACE inhibitors are characterized by the presence of an ionizable carboxylic group. The pK_a values of carboxylic acid fall in the range of 2.5 to 4.0, and therefore, the use of an alkaline buffer was considered.

With the exception of fosinopril, all ACE inhibitors possess a basic substituent in addition to the carboxylic acid group. The pK_a values for the basic amine function fall in the range of 5.0 to 10.1. As a consequence of the pH of the medium, the inhibitors are either mainly negatively, or positively, charged. This offers the possibility of using an acidic or an alkaline running buffer. The only pK_a values mentioned in the literature are those for enalapril and lisinopril [22,23]. The pK_a values for the other substances were calculated with ACD/pK_a DB (V3.5

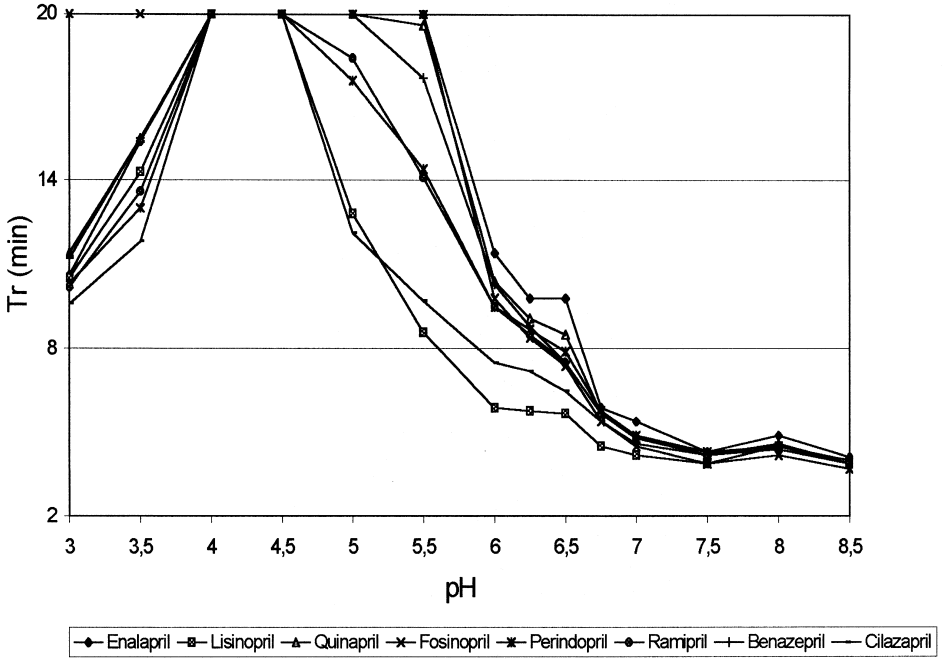

Fig. 2. Influence of the pH. Experiments were carried out using a fused-silica capillary 60 cm (52.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (50 m*M*) at varying pH as the running buffer, an applied voltage of 25 kV, and a detection wavelength of 214 nm. Tr=Migration time.

for Microsoft Windows, ACD/Labs Software; see Table 1).

The pK_a values of enalapril, quinapril, fosinopril, perindopril, ramipril, benazepril and cilazapril fall in the same range. Lisinopril is an exception because it possesses four pK_a values (pK_a 2.5 and 4.0 for the two carboxyl groups, and pK_a 6.7 and 10.1 for the primary and secondary amine groups). Thus, in principle, lisinopril can exist in five different forms in different pH buffers (dication, cationic zwitterion, zwitterion, anionic zwitterion, dianion) [23]. All the other ACE inhibitors, except fosinopril, can exist in only three different forms (cation, zwitterion, anion), so it is to be expected that lisinopril will be have in a different way to the others. Fosinopril is the only phosphorus-containing ACE inhibitor without a basic function (pK_a 3.8±0.6), so its behavior will also be different to the others. Fosinopril can only exist in two different forms, uncharged or anionic. There is therefore no difficulty in separating lisinopril and fosinopril from the other ACE inhibitors.

Table 1
pK_a values of ACE inhibitors

ACE inhibitor	pK_a value	
	Acid function	Basic function
Enalapril	3.0[a]	5.4[a]
	3.8±0.4	5.5±0.4
Lisinopril	2.2±0.1; 3.8±0.4	7.6±0.4; 10.5±0.1
	2.5[a]; 4.0[a]	6.7[a]; 10.1[a]
Quinapril	3.3±0.4	5.4±0.4
Fosinopril	3.8±0.6	–
Perindopril	3.7±0.4	5.7±0.4
Ramipril	3.7±0.4	5.5±0.4
Benazepril	3.7±0.1	5.0±0.4
Cilazapril	3.3±0.4	5.9±0.2

[a] pK_a values mentioned in the literature [22,23].

Below pH 2.0, all the ACE inhibitors possess good peak symmetry; however, they co-elute because they all have a positive charge. Conversely, fosinopril is uncharged and migrates with the marker. Between pH 2.0 and 3.5, the shape of the peak for both enalapril and perindopril becomes less symmetrical, and the migration time of fosinopril increases to more than 20 min. Only at pH 3.0 is it possible to

obtain a baseline separation between cilazapril, ramipril, lisinopril, and benazepril or quinapril. This pH is therefore only suitable for four ACE inhibitors. As already mentioned above, the pK_a values of the carboxylic acid group of those four ACE inhibitors fall in the range of 2.5 to 3.8 and it is all known that the biggest numerical difference in mobility is achieved in the pH area around the pK_a value.

Between pH 4.0 and 4.5, the ACE inhibitors become zwitterions with a net charge of zero (neutral compounds), and consequently their migration times become too long.

Between pH 5.0 and 8.5, the shape of the peak improves gradually; however, from pH 7.5 onwards, the selectivity decreases. This results in an inadequate separation, because all species become negatively charged and consequently co-elute from pH 8.0 onwards. The peak symmetry for all compounds is acceptable from pH 6.25, so the optimum pH for separation had to be sought in the range from 6.25 to 7.0. However, it remains impossible to separate all species because there are always some that co-elute. The length of the capillary also influences the separation; by using a capillary of 93 cm, instead of 60 cm, some compounds attained baseline separation in the longer capillary whereas in the shorter one, the same compounds could not be baseline-separated.

The optimum conditions for the separation of the majority of these compounds were found by using a sodium phosphate buffer (pH 7.0; 100 m*M*; see Fig. 3). Under these circumstances, it was possible to separate five compounds, namely lisinopril, fosinopril, cilazapril, enalapril, and one of the following combination: ramipril/quinapril/benazepril/perindopril (Fig. 4). Benazepril and enalapril on the one hand, and perindopril and enalapril on the other, could not be baseline-separated completely.

The co-eluting compounds ramipril, quinapril, benazepril, and perindopril can be divided into two groups: ramipril and quinapril on the one hand, and benazepril and perindopril on the other. Ramipril and quinapril co-elute at pH 7.0, but can be separated by using a sodium phosphate buffer (pH 6.25; 100 m*M*; see Fig. 5). Benazepril and perindopril can also be

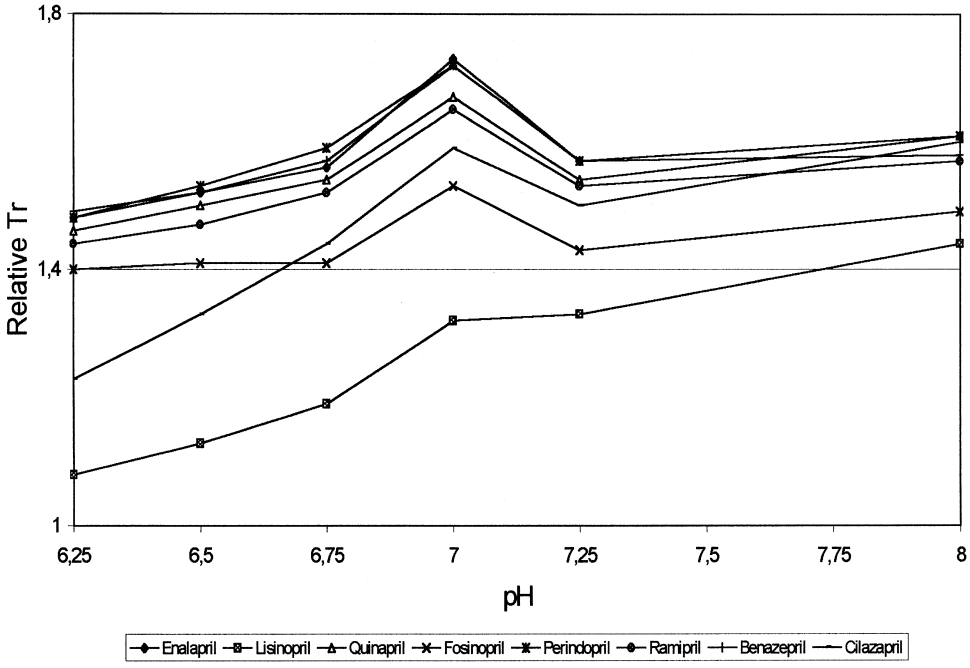

Fig. 3. Influence of pH in a small domain. Experiments were carried out using a fused-silica capillary 93 cm (85.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (100 m*M*) at varying pH as the running buffer, an applied voltage of 30 kV, and detection wavelength of 214 nm.

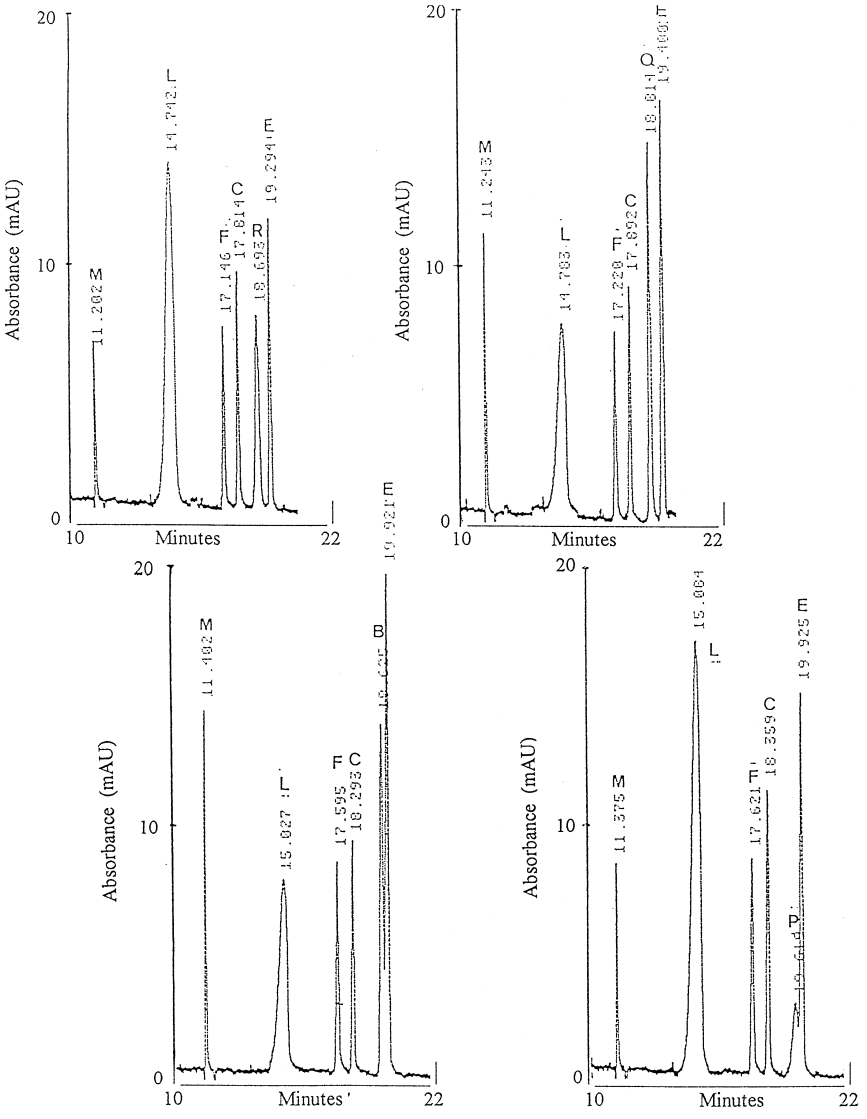

Fig. 4. Electropherogram of a mixture of several ACE inhibitors using a fused-silica capillary 93 cm (85.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 7.0; 100 m*M*) as the running buffer. The applied voltage is 30 kV and detection is at 214 nm. M: Marker (formamide); E, L, Q, F, P, R, B and C refer to the first letter of the corresponding ACE inhibitors.

separated by using a sodium phosphate buffer (pH 6.25; 100 m*M*; see Fig. 5). Under these circumstances, benazepril co-elutes with enalapril, but perindopril can be baseline-separated from enalapril (Fig. 5).

The choice of the pH of the running buffer is a critical factor for an adequate separation. The migration time and the peak symmetry of the different ACE inhibitors are greatly influenced by the pH of the medium (Fig. 2).

3.1.2. Influence of molarity

As well as pH, the ionic strength of the running buffer is another parameter that controls the retention of these compounds. We varied the molarity of the sodium phosphate buffer (pH 7.0) from 50 m*M* to

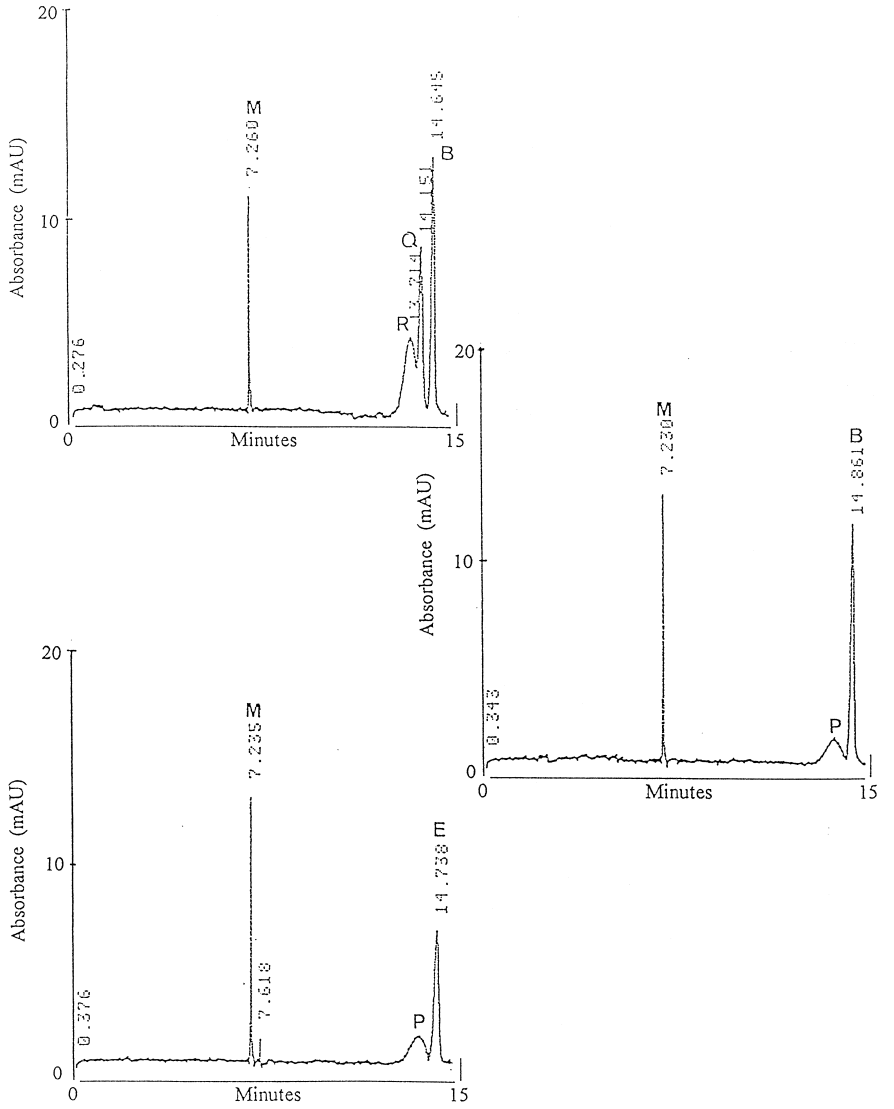

Fig. 5. Electropherogram of a mixture of several ACE inhibitors using a fused-silica capillary 93 cm (85.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 6.25; 100 m*M*) as the running buffer. The applied voltage is 30 kV and detection is at 214 nm. M: Marker (formamide); E, Q, P, R and B refer to the first letter of the corresponding ACE inhibitors.

150 m*M*. As already stated above, the best separation was obtained with a 100 m*M* sodium phosphate buffer (Fig. 6). When using a concentration below 100 m*M*, some compounds could not be baseline-separated, and when using a concentration above 100 m*M*, the migration times became longer, more baseline fluctuations occurred, and the degree of separation remained the same.

Since the simple capillary zone electrophoresis (CZE) mode could not solve the problem of the separation of all the ACE inhibitors in one run, we tried micellar electrokinetic chromatography (MEKC).

3.2. MEKC

3.2.1. Influence of sodium dodecyl sulfate

Since sodium dodecyl sulfate (SDS) is widely

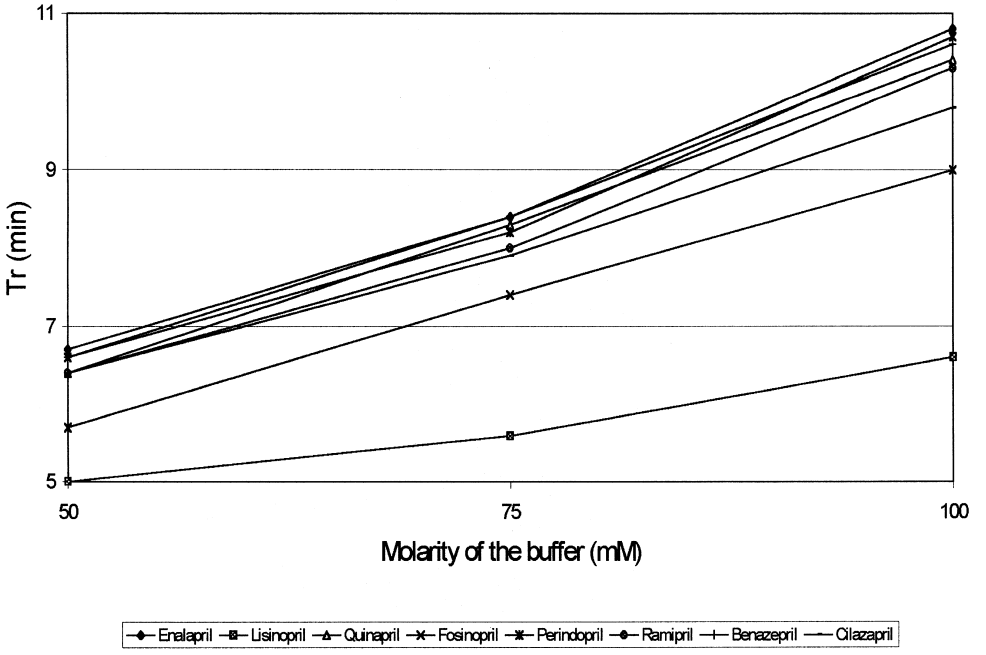

Fig. 6. Influence of buffer molarity. Experiments were carried out using a fused-silica capillary 60 cm (52.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 7.0) as the running buffer, an applied voltage of 20 kV, and detection wavelength of 214 nm.

used in MEKC, the influence of SDS on inhibitor separation was examined in detail. Although we know that SDS should be applied at a pH around and above 9 for maximal hydrophobic interaction, SDS was not used by that pH. Most of the ACE inhibitors are esters so stability problems occur in a strong alkaline medium. SDS was therefore used only at pH 7.0 and pH 8.0. When using SDS in a concentration higher than 10 m*M*, the peaks for enalapril, perindopril, ramipril, and quinapril became asymmetric and sometimes peak splitting occurred. This effect was independent of the pH of the running buffer.

The system was found to be suitable only for four ACE inhibitors, namely lisinopril, fosinopril, benazepril and cilazapril. An apparent increase of selectivity was observed only at pH 8.0, because the four compounds were co-eluting without the addition of SDS (Fig. 7). At pH 8.0, the addition of SDS improved the separation of some of the drugs. Only a ternary mixture of lisinopril, fosinopril and benazepril or cilazapril could be separated; however, these same compounds could already be separated using a sodium phosphate buffer (100 m*M*; pH 7.0). Under these latter conditions, it was possible to separate five compounds, so the use of SDS was therefore unable to improve the overall separation.

In summary, the selectivity of separation is affected by the addition of SDS to the running buffer (pH 8.0; 100 m*M*); however, owing to the negative influence of SDS on the peak symmetry, it cannot be used for all compounds. When using a combination of parameters such as SDS and organic solvents, the peak shape remained asymmetrical and baseline fluctuations appeared.

In summary, MEKC could not solve the problem of the separation of all eight compounds and resulted in no net improvement in separation, compared to CZE.

4. Conclusion

The above results prove that the capillary electrophoretic separation of the eight ACE inhibitors cannot be achieved by using a single system. A combination is necessary for the selective identification of the structurally related substances with their similar pK_a values. The optimum conditions for the

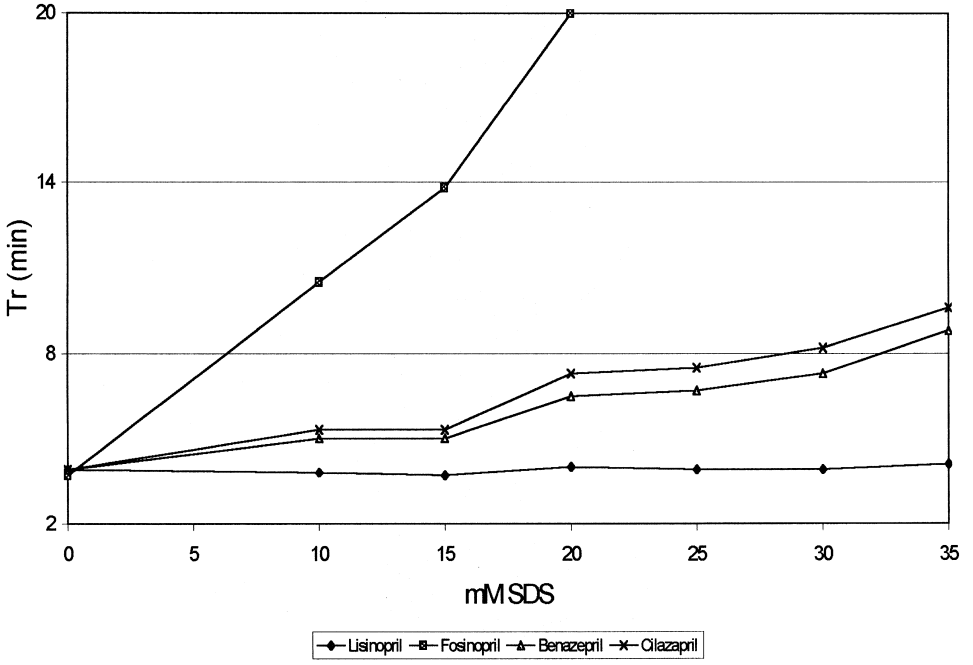

Fig. 7. Influence of SDS. Experiments were carried out using a fused-silica capillary 60 cm (52.5 cm to the detector)×50 μm I.D., and sodium phosphate buffer (pH 8.0; 50 m*M*) as the running buffer, an applied voltage of 25 kV, and detection wavelength of 214 nm.

separation of most of these compounds are attained by employing a sodium phosphate buffer (pH 7.0; 100 m*M*). Under these conditions, it is possible to separate five compounds, namely lisinopril, fosinopril, cilazapril, enalapril, and one of the following drugs in the combination ramipril/quinapril/benazepril/perindopril. The co-eluting compounds ramipril, quinapril, benazepril and perindopril can then be distinguished by using a second system, also employing a sodium phosphate buffer, but at a different pH (pH 6.25; 100 m*M*). The combination of the two systems can be applied successfully to the identification of these compounds in pharmaceutical formulations, the identification being possible on the basis of the migration time using CE.

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