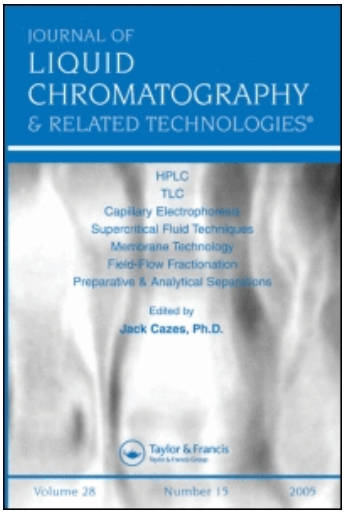

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Separation of Lisinopril and Its RSS Diastereoisomer by Micellar Electrokinetic Chromatography

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SEPARATION OF LISINOPRIL AND ITS RSS DIASTEREOISOMER BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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ABSTRACT

Diastereoisomer separation of pharmaceuticals having several chiral centers by capillary electrophoresis is exemplified by the separation of lisinopril (SSS) (an inhibitor of angiotensin converting enzyme) from its RSS diastereoisomer. Using micellar electrokinetic capillary chromatography (MECC), excellent resolution between the two diastereoisomers was achieved by using a bile salt, sodium cholate, as the surfactant in electrolyte under optimized pH, organic content and temperature conditions. The RSS compound eluted earlier than the SSS compound. A good separation was also achieved by the use of sodium dodecyl sulfate (SDS) as a surfactant in electrolyte, but the eluting sequence was reversed, resulting from the different hydrophobic/hydrophilic nature of the two surfactants.

INTRODUCTION

Lisinopril (SSS) **1** is the dihydrate of 1-[N^2- (1(*S*) - carboxy-3-phenylpropyl)-L-lysyl]-L-proline, an important long-acting angiotensin

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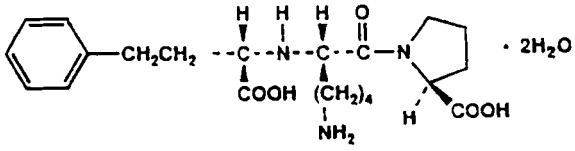

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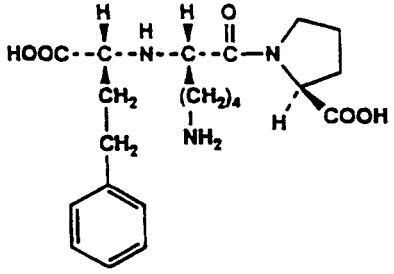

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converting enzyme inhibitor for the treatment of hypertension [1]. Lisinopril has three chiral centers, and thus eight diastereoisomers. The synthetic sequence for the preparation of lisinopril developed by Merck Research Laboratories yields lisinopril containing less than 1% RSS diastereoisomer **2**. A good separation of lisinopril and its RSS diastereoisomer is essential to assess the purity of lisinopril bulk drug.

Since rotation around the amide bonds is hindered at room temperature, both the SSS and the RSS compounds can exist in cis and trans rotamers in solution, and the *cis-trans* interconversion, like other proline-containing dipeptides, has relaxation times in the order of minutes [2,3]. Due to the slow *cis-trans* interconversion, broadening or splitting of the lisinopril peak was

observed in HPLC [1]. Consequently, good separation of SSS and RSS diastereoisomers by HPLC was difficult to achieve.

Recently, micellar electrokinetic capillary chromatography (MECC) has been demonstrated as a highly efficient separation technique [4,5,6]. In the present study, this technique is applied to the separation of lisinopril (**1**) and its RSS diastereoisomer (**2**). More recently, the use of bile salts as surfactants in the electrolytes of MECC has proved very effective for the separation of chiral stereoisomers [7-12]. By using sodium cholate as the surfactant as well as by examining the effects of pH, organic content, and temperature of the electrolyte on resolution, an excellent separation of **1** and **2** (resolution about 2.6) was achieved. A good separation was also obtained by the use of sodium dodecyl sulphate (SDS) as a surfactant. A significant observation is that the RSS compound eluted earlier than the SSS compound when sodium cholate was used, while the elution order was reversed when SDS was used. Difference in the hydrophobic/hydrophilic nature of the micelles formed from these two surfactants is most likely the cause. To the best of our knowledge, this is the first example demonstrating the MECC separation of diastereoisomers possessing cis & trans rotamers. This study serves as an excellent model for the separation of many other important drugs possessing more than one chiral centers and having rotamers (e.g., enalapril, captopril etc.).

The separation of **1** and **2** by bile-salt MECC shows several special features as compared to the separation of planar molecules, which could be significant in the further development of bile-salt MECC. Nishi et al. [9,10] and Sepaniak et al. [11,12] observed that pH of the electrolyte and the rigidity of the solute structures are two important factors which influence the chiral separation in bile-salt MECC. It appears that the bile salts prefer a rigid, planar structure for chiral

recognition, which is probably due to their rigid structures [8]. However, the structures of lisinopril and its RSS diastereoisomer are not rigid, because they have interconvertable rotamers. The good MECC separation of these compounds reported here provides an example of the separation of non-rigid diastereoisomers by bile-salt MECC. Cole and Sepaniak [11] pointed out that reducing the pH of the electrolyte to eliminate the coulombic repulsion between solute and micelle is critical to achieving chiral recognition with bile salts. On the contrary, an excellent separation of **1** and **2** was achieved at pH 9.55 electrolyte, demonstrating that the coulombic repulsion does not inhibit or even facilitate the separation of these diastereoisomers. Furthermore, it is shown in this study that since the interconversion rates of the rotamers are temperature-dependent [2,3], temperature is another critical factor in addition to pH and structure in the separation of lisinopril and the RSS diastereoisomer by bile-salt MECC.

EXPERIMENTAL

Instrumentation and procedure for electrophoresis

This study was performed on an Applied Biosystems Instrument Model 270-HT (Applied Biosystems, San Jose, California) equipped with a fused-silica capillary 72 cm in total length (50 cm from inlet to detector) and 50 μm in internal diameter. The capillary is enclosed in a thermostatically controlled compartment which can be controlled from room temperature (26 °C) to 60 ± 0.1 °C. Hydrodynamic injection (5" Hg) was employed with typical injection times of 2 seconds. A constant potential of 30 kV was used for the study. Ultraviolet absorbance at 210 nm was employed for detection.

Before analysis, fused-silica capillaries were prepared by successive washing with 0.1M sodium hydroxide, distilled water, and finally the running

electrolyte. Between runs, the capillary was always flushed with the running electrolyte.

Chemicals and Sample Preparation

Pharmaceutical grade lisinopril and its RSS diastereoisomers were manufactured within Merck Research Laboratories (Rahway, New Jersey). Cholic acid (sodium salt hydrate (98%)), deoxycholic acid (sodium salt monohydrate (98%)), sodium dodecyl phosphate (99%), α and β–cyclodextrin hydrate, and tris(hydroxymethyl)aminomethane (99.9%) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). Methanol (OPTIMA grade), sodium hydroxide (0.1M solution) and phosphoric acid (85%, HPLC grade) were purchased from Fisher Scientific Company (Philadelphia, Pennsylvania). They were all used as received without further purification. Deionized water with at least 18 M-Ohm purified by a Milli-Q (Bedford, Massachusetts) system was used for the preparations of electrolytes and samples.

The samples of lisinopril and the RSS compound were prepared by dissolving 10 mg lisinopril (or the RSS compound) in 100 ml water (concentration: 0.1 mg/ml). The 1:1 mixture sample was prepared by mixing 10 ml each of the lisinopril and the RSS samples.

RESULTS AND DISCUSSION

1. Different Ions of Lisinopril

At 25 °C, the aqueous acidic/basic potentiometric titration of lisinopril yielded four pK_a values of 2.5, 4.0, 6.7 and 10.1 [1]. Among them, 2.5 and 4.0

are the pK_a values of the two carboxyl groups, and 6.7, 10.1 are those of the primary and the secondary amine groups, respectively. Thus, in principle, lisinopril can exist in five different forms in different pH buffers. According to these pK_a values, lisinopril forms a dication (both amine groups are protonated) below pH of 2.5. Between pH 2.5 and 4.0, lisinopril becomes a cationic zwitterion with a net charge of +1 (one carboxyl group is ionized). It becomes a zwitterion with a net charge of zero (a neutral compound) between pH 4.0 and 6.7 because both carboxyl groups can be ionized in this pH range. Between pH 6.7 and 10.1, lisinopril can form an anionic zwitterion with a net charge of -1 (both carboxyl groups are ionized and only the primary amine group is protonated). Above pH 10.1, since the two carboxyl groups are ionized and two amine groups are deprotonated, lisinopril can form a dianion. No pK_a values of the RSS compound have been reported in the literature. However, it is reasonable to assume that the pK_a values of the RSS compound are similar to those of lisinopril. The actual pK_a values of lisinopril in electrolytes may be slightly different from those obtained by the aqueous acidic/basic potentiometric titration, and thus the exact range in which different ions of lisinopril are formed may slightly differ from the above.

Since the capillary silica can dissolve in solutions below pH 2.5 and above pH 10, these pHs were not used in the study. Although MECC is a well-documented technique for separation of neutral species [4-12], it was observed that some neutral species (e.g., methanol) co-eluted with lisinopril in the pH range between 4.0 and 6.7. Therefore, this pH range was also not applicable. The chosen conditions were those under which either monocationic (pH 2.5 to 4.0) or monoanionic (pH 6.7 to 10.1) lisinopril is formed. In this report, the pH

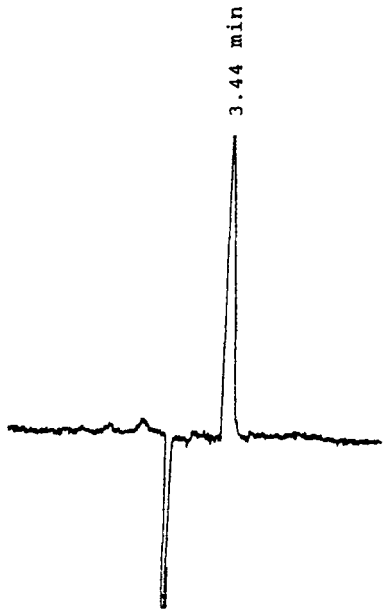

Figure 1 Electropherogram of lisinopril (SSS) and its RSS diastereoisomer. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 25 mM tris-PO_4, pH 9.55; applied voltage: 30 kV; 30 °C.

conditions for the formation of monoanionic lisinopril were mainly investigated. Since the pH conditions for the formation of monocationic lisinopril seemed more complicated (see below), the study of monocations were only briefly described for comparison.

2. CZE Separation of the SSS and RSS Diastereoisomers

The separation of lisinopril (SSS) and the RSS diastereoisomers was first attempted by capillary zone electrophoresis (CZE). Using an electrolyte

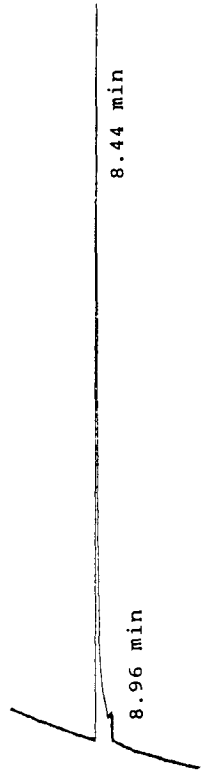

Figure 2 Electropherogram of lisinopril (SSS). Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 25 mM tris-PO_4, pH 2.7; applied voltage: 30 kV; 30 °C.

composed of 25 mM tris-PO_4, pH 9.55 (30 °C), both SSS and RSS diastereoisomers form the corresponding monoanions. These monoanions co-migrated at 3.4 min. as shown in Figure 1. This is expected since the anions of the two compounds have the same charge to mass ratio.

The separation of SSS and RSS diastereoisomers was also attempted by CZE using acidic buffers (25 mM tris-PO_4, pH 2.7) as the electrolyte, in which

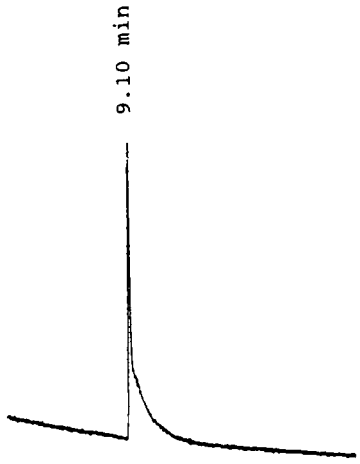

Figure 3 Electropherogram of the RSS diastereoisomer. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 25 mM tris-PO_4, pH 2.7; applied voltage: 30 kV; 30 °C.

both compounds can form monocations. Interestingly, the lisinopril SSS cation showed a large peak at 8.44 min. and a small peak at 8.96 min. (Figure 2). Upon comparison with the electropherograms of cis and trans rotamers of enalapril [13], these peaks were assigned to the cis and trans rotamers of the lisinopril cation. Under the same conditions, the RSS cation showed only one peak at 9.10 min. with fairly large tailing (Figure 3). The rotamers were unresolved. The difference in observation between the two diastereoisomers under these conditions may be attributed to different cis-trans interconversion rates of the cations [13].

It should be pointed out that the lisinopril (SSS) and the RSS cations showed slightly different migration times, probably due to the different interactions between the hydrophobic groups of the two cations and the silica

capillary wall under acidic conditions. But, because the separation was complicated by the distortion of peaks due to rotamers, further studies were conducted under basic conditions.

3. Sodium cholate MECC Separation of the SSS and RSS Diastereoisomers - Effect of Methanol Content on the Separation

Since simple CZE did not succeed in the separation of the SSS and RSS monoanions, MECC was attempted. Sodium cholate was selected as a surfactant in the electrolyte for the reasons mentioned in the Introduction. The critical micelle concentration of this surfactant is about 15 mM. Using a buffer (25 mM tris-PO_4) doped with 50 mM sodium cholate at pH 9.55, the SSS and RSS monoanions were slightly separated as shown in Figure 4.

An effort was made to improve the separation. It is well-known that addition of the proper amount of methanol (or acetonitrile and other organic compounds) into the electrolyte often permits the optimal k' for maximum resolution. Fanali [14] demonstrated that the best resolution for the separation of enantiomers of propanolol by cyclodextrins could be obtained by adding 30% methanol. In the present study, the resolution (R_S) between the lisinopril and the RSS diastereoisomer peaks as a function of the methanol content was carefully studied. The results are shown in Figure 5, in which, R_S was calculated by the usual formula: $R_S = 2\,(t_1 - t_2)/(w_1 + w_2)$, where t_1 and t_2 are the migration times of lisinopril and its RSS diastereoisomer and $(w_1 + w_2)/2$ is the average peak width at the peak base, respectively. Dramatic improvement of the resolution was observed when the methanol content increased to above 45%. The best separation (resolution about 2.5) was observed at a methanol content of 55%.

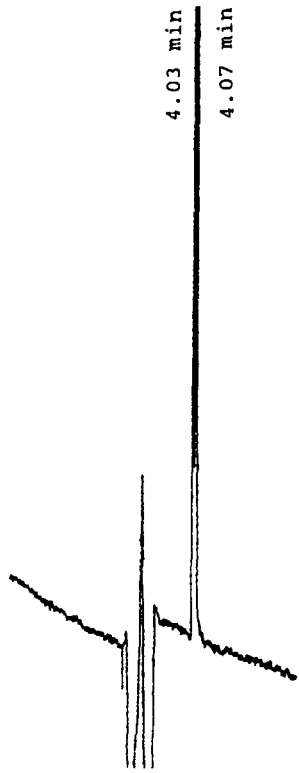

Figure 4 Electropherogram of lisinopril (SSS) and its RSS diastereoisomer using sodium cholate as the surfactant. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 25 mM tris-PO_4, 50 mM sodium cholate, pH 9.55; applied voltage: 30 kV; 30 °C.

Further increase of the methanol% resulted in decreased resolution. These results are rather surprising since the % methanol reported in the literature is usually less than 30%. The role of organic solvents in bile-salt MECC has been well explained [11]. Particularly, it has been studied by examining the impact of methanol content on micelle formation [15]. It was found that the critical micelle

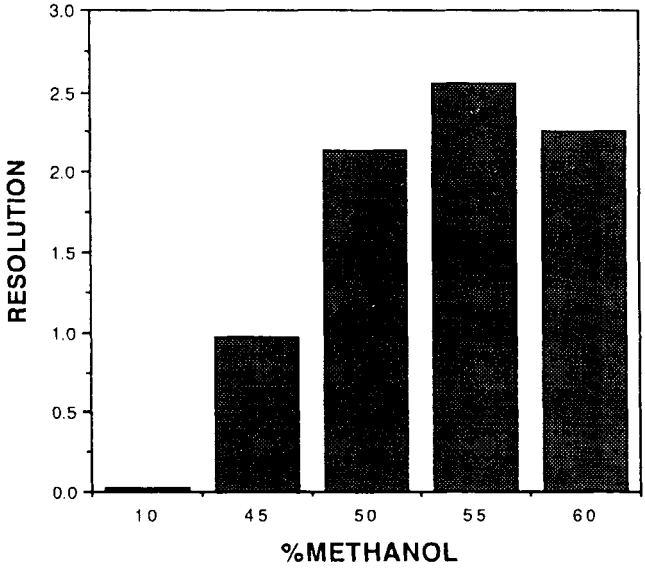

Figure 5 Resolution of lisinopril and its RSS diastereoisomer as a function of methanol content. Other conditions were the same as in Figure 4.

concentration (CMC) for sodium dodecyl sulphate (SDS) begins to rise at methanol levels above about 10%, while the CMC for sodium cholate stays nearly constant until 30% methanol. However, above 30% methanol, the CMC should rise. These studies indicate that although the concentration of sodium cholate used in the present study is high (50 mM), the break down of the bile-salt micelle into dimers and/or oligomers at 55% methanol is highly possible. Nevertheless, the experiments show that the best separation was achieved at 55% methanol. Therefore, there is a possibility that the separation can be improved by the interaction between lisinopril and its RSS diastereoisomer with dimers and/or oligomers of sodium cholate.

4. pH Effect on the Separation by the Sodium Cholate - MECC

As shown above, the excellent separation of the monoanions of the SSS and RSS diastereoisomers were achieved by the use of sodium cholate and 55% methanol at pH 9.55. It has long been recognized that the buffer pH significantly affects the recognition of enantiomers [9,10]. The pH is particularly critical in the present study since both lisinopril and its RSS diastereoisomer can exist in as many as five different forms under the different pH conditions discussed previously. Using 45% buffer (25 mM tris-PO_4 and 50 mM sodium cholate) and 55% methanol (v:v)) at 30 °C, the pH effect on the separation was more carefully studied under the basic conditions. As shown in Figure 6, the separation was poor at pH 8.25. As pH increased, the migration times of both SSS and RSS anions increased, and the resolution improved. At pH 9.55, the best resolution (about 2.6) was achieved. It is noteworthy that this pH is close to the highest pK_a of lisinopril (10.1). The experiments showed that even in the pH range (6.7 to 10.1) in which lisinopril (SSS) and its RSS diastereoisomer become the corresponding monoanions, a difference in pH could make a difference in separation. Since the pH of the buffer changes the % monoanion formation from both the lisinopril and the RSS molecules as well as the ionic states of the bile salt micelles (the bile salts form anionic micelles by the hydrophobic interactions between the non-polar faces of the monomers [16]), the pH can change the interactions between these two anions and the anionic bile-salt micelles due to charge effects. For the separation of the monoanions of lisinopril and the RSS compound, buffers with pHs 9.0 to 9.6 were found to be most suitable. This result is significant since nearly all enantiomeric separations by bile-salts involving negatively charged solutes have been unsuccessful [7,11]. The failure

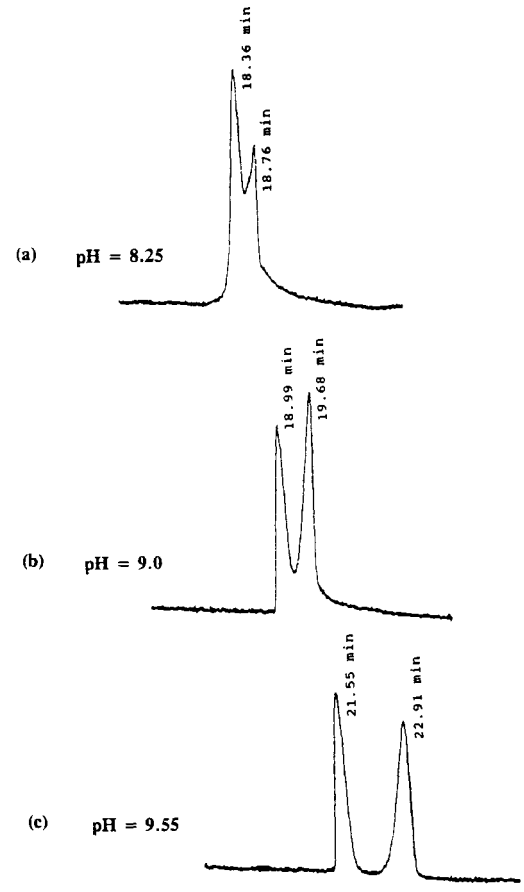

Figure 6 Effect of pH on the separation of lisinopril and its RSS diastereoisomer. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 45% 25 mM tris-PO_4 and 50 mM sodium cholate, 55% methanol (v:v); applied voltage: 30 kV; 30 °C.

of the anionic separation was attributed to coulombic repulsion between the anions and negatively charged bile salt micelles. The present study shows that coulombic repulsion does not inhibit the separation of diastereoisomers possessing several chiral centers like lisinopril.

Under anionic conditions, no rotamers of lisinopril and the RSS diastereoisomer were separated, probably because the interconversion of cis- and trans-rotamers of monoanions of these compounds is very fast.

5. Temperature Effect on the Separation by Bile-Salt MECC

The effect of temperature in MECC separations so far has not been addressed as extensively as pH and organic solvents in MECC, probably because temperature does not play as critical a role as does pH and organic solvents for rigid molecules. However, lisinopril and its RSS diastereoisomer are non-rigid molecules with interconvertable rotamers, therefore, temperature becomes significant in their separation. The optimization of the separation in terms of organic content and pH discussed above were performed at 30 °C. As temperature increased to 45°C, the peak of the SSS compound (late eluting peak) remained sharp, but the peak of the RSS compound (early eluting peak) became broadened and distorted. Particularly, extreme tailing of the RSS peak generally increased as temperature increased. The separation became worse at 45 °C as compared to that at 30 °C (Figure 7). A reasonable explanation is that there is an increased difference in mobility between the RSS compound and the buffer component [17,18] at higher temperatures. It appears that lower temperature favors the separation of the monoanions of the two diastereoisomers by sodium cholate - MECC. Lower temperature was also favored in chiral separations by

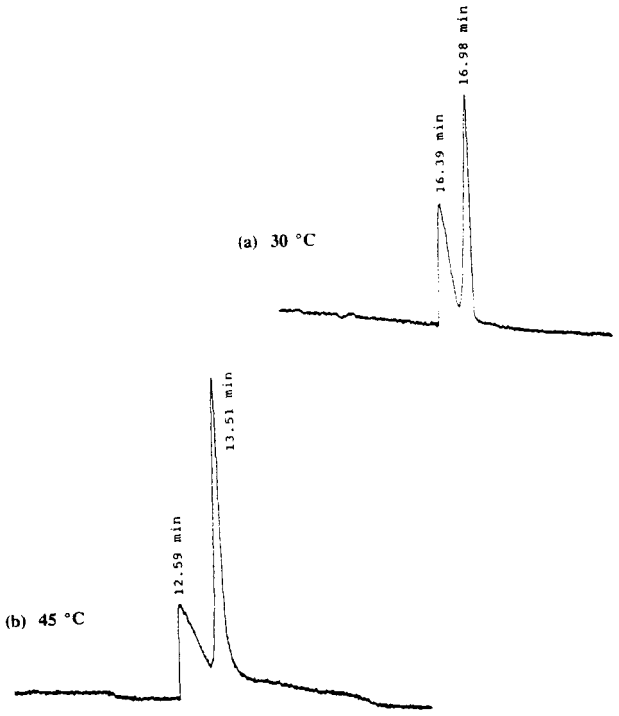

Figure 7 Effect of temperature on the separation of lisinopril and its RSS diastereoisomer. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 50% 25 mM tris-PO_4 and 50 mM sodium cholate, 50% methanol (v:v), pH 9.55; applied voltage: 30 kV.

cyclodextrins as reported by Altria et al. [19]. It is noteworthy that although HPLC methods for the determination of lisinopril under acidic conditions (e.g. pH 2) [1,20] have been extensively studied, no HPLC method under basic conditions has been developed and reported so far. This study demonstrates the temperature effect for the separation of lisinopril and the RSS diastereoisomer under basic conditions for the first time.

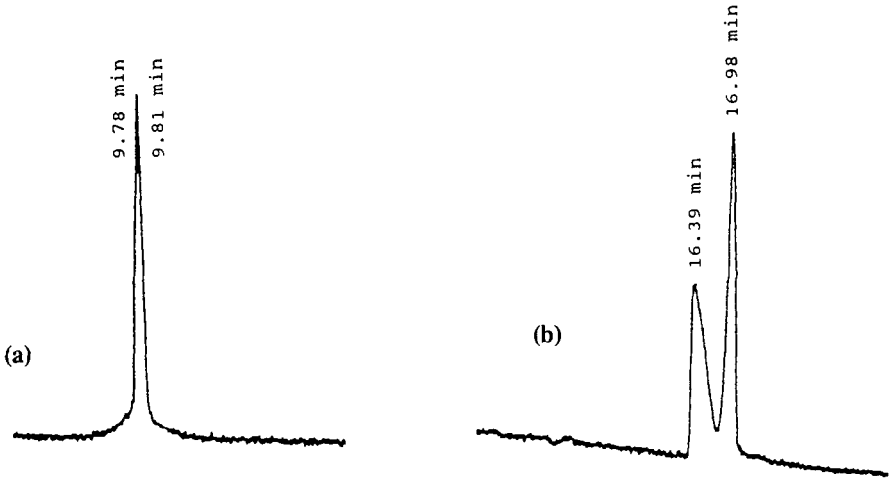

Figure 8 MECC separation of lisinopril and its RSS diastereoisomer using (a) α–cyclodextrin and (b) sodium cholate as surfactants. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); applied voltage: 30 kV; 30 °C; electrolyte: (a) 50% 25 mM tris-PO_4 and 30 mM α-cyclodextrin, 50% methanol (v:v), pH 9.55, (b) 50% 25 mM tris-PO_4 and 50 mM sodium cholate, 50% methanol (v:v), pH 9.55.

6. Comparison of Cyclodextrins with Bile Salts

Among the most successful chiral additives are cyclodextrins, which have been used to separate optical isomers of many pharmaceuticals [14, 19, 21-25]. In the present study, it was observed that using α-cyclodextrin in the electrolyte (50% 25 mM tris-PO_4 and 30 mM α-cyclodextrins, 50% methanol (v:v), pH =9.55), only slight separation of the SSS and RSS diastereoisomers were achieved (Figure 8a). β-Cyclodextrin and γ-cyclodextrin were also attempted under similar conditions. The separation was not much improved. The 30 mM α–cyclodextrin used in the study is a reasonable concentration used in the chiral

separation [19, 21-25]. However, our experiments show that the separation by cyclodextrins was much worse than that by sodium cholate under the same conditions (see Figure 8b). Since the separation by cyclodextrins is mainly due to the host-guest complexation of the solutes and the cyclodextrins [26], it seems that the size of the cavity and the hydrophobic nature of the cyclodextrins were not suitable for the formation of host-guest complexes between the non-rigid lisinopril and cyclodextrins or between the RSS diastereoisomer and cyclodextrins.

7. Comparison of SDS with Bile Salts

As shown above, bile-salt MECC was successfully applied to the separation of diastereoisomers having more than one chiral center and rotamer. In addition to sodium cholate, another commonly used bile salt, sodium deoxycholate, was also found to be suitable for the separation of lisinopril and its RSS diastereoisomer. However, under the same conditions, the separation was much better if sodium cholate was used. Sodium deoxycholate is a dihydroxy steroid while sodium cholate is a trihydroxy steroid. Both these bile salts, owing to the presence of hydrophilic hydroxyl groups, are relatively polar and, consequently, provide lower k' values for most solutes relative to sodium dodecyl sulfate (SDS). However, sodium cholate, having a higher degree of hydroxyl substitution, can yield lower k' values than sodium deoxylcholate. Since lisinopril and the RSS compound are moderately hydrophobic compounds, sodium cholate, due to its more hydrophobic nature, is a better choice in the present study [11].

SDS is probably the most commonly used surfactant in MECC. However, since SDS is not a chiral surfactant, it cannot be applied to the separation of

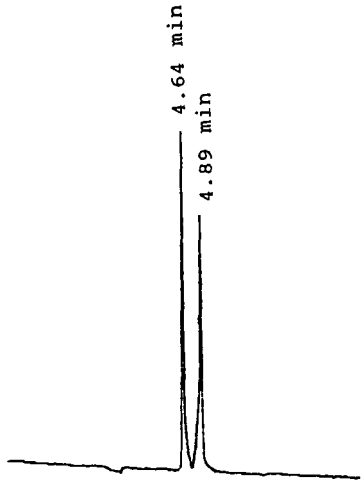

Figure 9 MECC separation of lisinopril and its RSS diastereoisomer using SDS as the surfactant. Conditions: fused silica capillary (72 cm (50 cm to detector), 50 μm i.d.); electrolyte: 25 mM sodium phosphate and 40 mM SDS, pH 9.0; applied voltage: 30 kV; 30 °C.

enantiomers without other chiral additives. Lisinopril and the RSS compound are chiral compounds but they are also diastereoisomers which possess different physical and chemical properties. As a result, the separation of these two compounds was attempted by SDS - MECC under anionic conditions. As shown in Figure 9, using 20 mM sodium phosphate (pH 9.0) and 40 mM SDS as the electrolyte, a base-line separation of the anions of these two compounds was obtained. When temperature was increased, the peaks of both compounds became broadened, worsening the separation, the observation being similar to that observed by the use of bile-salt MECC. However, there is one important difference between SDS and bile salt separation. In SDS - MECC, lisinopril (SSS) eluted earlier than the RSS diastereoisomer; while in bile - salt MECC, the

RSS compound eluted first. The eluting sequences were verified by studying the individual diastereoisomer under identical conditions. Since lisinopril and the RSS form monoanions at pH 9 to 9.6 in both SDS-containing and bile salt-containing buffers, it is unlikely that the observed sequences are due to the different ionic states of lisinopril or its RSS diastereoisomer. But it is well-known that bile salt aggregates are more polar than those formed with n-alkyl surfactants like SDS [12, 26]. Therefore, the different eluting sequences could be attributed to the different hydrophobic/hydrophilic natures of the micelles formed from these two surfactants.

CONCLUSION

The separation of lisinopril (SSS) and its RSS diastereoisomer was investigated by MECC. An excellent separation (resolution about 2.6) was achieved by using sodium cholate as a surfactant in the electrolyte after optimizing pH, organic content and temperature. A good separation was also achieved by using SDS as the surfactant. This appears to be the first example of separating non-rigid diastereoisomers having multiple chiral centers by MECC. Many other drugs (e.g., enalapril, captopril, etc.) are also non-rigid compounds similar to lisinopril. This study demonstrates that MECC can be successfully applied to the separation of these compounds, achieving excellent resolution.

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