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Study of the *cis–trans* isomerization of enalapril by reversed-phase liquid chromatography

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Abstract

Enalapril is a dipeptidic angiotensin converting enzyme inhibitor. It exists as a mixture of two conformers in solution with respect to the peptide bond involving the proline amino group. The RPLC of such products may yield peak splitting or multiple peaks as a result of the slow kinetics of the conformation change. In this study, the influence of the flow-rate, pH, temperature, organic modifier and counter ion on the peak shape and the separation of the *cis* and *trans* conformers are examined qualitatively by HPLC. It appears that decrease of relaxation time for isomerization with concomitant improvement in peak shape is favoured by a decrease in pH and flow-rate, increase of temperature, choice of organic solvent (nature, amount) and cationic counter ion concentration in the mobile phase. The elution order of the isomers was dependent on the nature of the organic modifier whereas the separation selectivity was improved by an increase of pH or the addition of a negatively charged counter ion. In addition, an NMR investigation on enalapril is described.

Keywords: Positional isomer separation; Enalapril; Enzyme inhibition

1. Introduction

Enalapril {L-proline, 1-[N-(ethoxycarboxyl)-3-phenylpropyl]-L-alanyl]-(S)}, a potent angiotensin converting enzyme (ACE) inhibitor, is an effective antihypertensive drug [1]. The appearance of multiple peak in reversed-phase liquid chromatography (RPLC) of dipeptides containing L-proline have been observed [2,3]. On the other hand, for captopril and ramiprilate, the NMR studies revealed the existence of two isomers for each product with respect to the conformation across the amide bond [3–5]. In addition it was reported that such conformational change has relaxation times in the order of minutes [6–8]. Since rotation around the amide bond occurs at room temperature, the enalapril exists also as a mixture of *cis* and *trans* conformers in solution. Both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used either for rotamer separation or the determination of enalapril [9–12].

However, to obtain a single sharp peak for quantitative analysis of enalapril by using HPLC, high temperature, low pH and counter ion are generally used. To our knowledge, no studies have described the influence of the nature of the organic modifier. In addition the effect of counter ion is still not well documented.

We therefore undertook this study to pursue these observations by examining qualitatively the various operating conditions on the retention peak, splitting

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and band broadening in order to provide more practical experimental conditions, allowing both the elution of enalapril as a single peak and its separation from impurities. We briefly describe the results of ^{1}H NMR of enalapril.

2. Experimental

2.1. Chromatography

Liquid chromatographic analyses were performed using a LC-10AD pump (Shimadzu) connected to a photodiode array detector (SPD-M10AV, Shimadzu) set at 215 nm. Injection was performed using a Rheodyne model 7725i injector (USA) valve with a 20-μl loop. The data were collected on a Packard Bell (USA) computer and the results were printed on a HP DeskJet 690 (Hewlett-Packard USA). The whole chromatographic system was controlled by the same computer, using Shimadzu LC-WORK STATION software. A Supelco LC 18 (5 μm) column (250× 4.6 mm I.D.) and a guard column (20×4.6 mm I.D.) both from Supelco (Bellefonte, PA, USA) were used. For the constant chromatographic column temperature control, a column oven (CTO-6 AS Shimadzu) was incorporated into the system. The pH of mobile phase buffers was adjusted by means of a model CG 825 pH meter from Schott (Germany).

2.2. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra were obtained at 300.13 MHz on a Bruker spectrometer. The probe temperature was 298 K. ^{1}H Chemicals shifts were measured relative to tetramethylsilane [TMS, $(CH_3)_4Si$]. The seal width spectra was 4201.68 Hz and relaxation time was 0.5 s. All measurements were made on enalapril in $C^2H_3SOC^2H_3$ solution.

2.3. Reagents and mobile phase

Enalapril maleate and its two main degradates; enalaprilat (Diacid) and diketopiperazine were kindly donated by MSD (USA). Potassium dihydrogenphosphate, sodium hydroxide and phosphoric acid were of R.P. quality from Prolabo (France). Methanol, acetonitrile and tetrahydrofuran (THF) were of HPLC grade, from Labscan (Dublin, Ireland). Octanesulfonic acid (sodium salt) and tetrabutylammonium hydrogensulfate, HPLC grade, were obtained from Fluka (Switzerland). Sample solutions were prepared by dissolving an appropriate amount of the corresponding substance in water to yield the concentrations of 1 mg/ml for diacid and 0.5 mg/ml for both enalapril maleate and diketopiperazine. The mixture solution of enalapril maleate, diacid and diketopiperazine was prepared by mixing and properly diluting in water the three sample solutions. All these preparations were stored at 4°C for a maximum of 2 weeks. The mobile phase consisted of 0.02 *M* phosphate buffer and an organic modifier (acetonitrile, methanol or THF) to which a counter ion was, in some cases, added. The pH values of the aqueous fraction of the eluent and in some cases the mobile phase were adjusted with phosphoric acid or sodium hydroxide (1 *M*) if necessary. The mobile phases were always filtered using 0.45-μm membrane filter (Supelco) and degassed by sonication. A 20-μl volume of a sample solution, of enalapril maleate (0.5 mg/ml) or the mixture solution of the three analytes was injected.

3. Results and discussion

3.1. Flow-rate

Chromatograms of enalapril obtained at four flow-rates 0.3, 0.5, 1 and 2 ml/min with a mobile phase consisting of a mixture of 20 m*M* phosphate buffer, pH 2–acetonitrile (60/40, v/v) at ambient temperature are shown in Fig. 1. At the lower flow-rate of 0.3 ml/min, a single peak ($t_R = 12.9$ min) is obtained, since enalapril has time to isomerize thus giving a broad band which relaxes. However the increasing of the flow-rate yields two distinct peaks or to be more precise a bimodal peak corresponding to the isomers due to the fact that enalapril has little time to isomerize. This change of the peak shape with a change in the flow-rate is in agreement with a reported study [3,7,8,13,14] on similar products having relaxation times of *cis–trans* isomerization commensurate to their retention times.

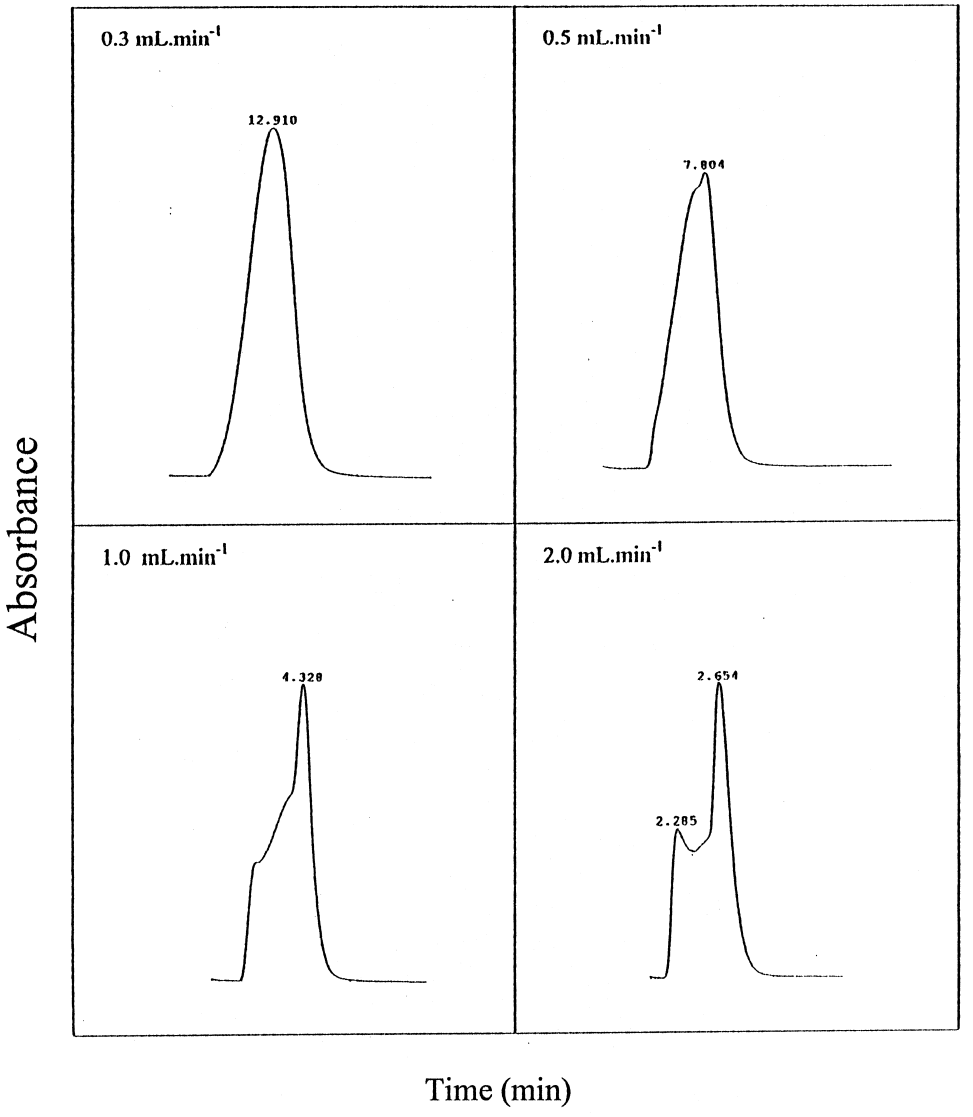

Fig. 1. Effect of flow-rate on the peak shape of enalapril; mobile phase: phosphate buffer, pH 2–acetonitrile (60:40, v/v); ambient temperature: stationary phase Supelco LC 18, 5 μm (250×4.6 mm I.D.).

3.2. Column temperature

The influence of the temperature on the retention time and the peak shape of enalapril was investigated at several column temperatures with the mobile phase previously used at a flow-rate of 1 ml/min. Fig. 2 shows that the increase of temperature led to a significant improvement in the peak shape. This effect is attributed to the acceleration of the conformational changes between the two isomers at higher temperatures thus giving a single peak. However, the effect of temperature on retention is less significant. The same results were obtained in a previous RPLC studies on proline-containing substances [3,8,15–17] and elevated column temperature was used for elution of enalapril as a single peak [10,18,19] whereas a low temperature is necessary for separation of isomers [10].

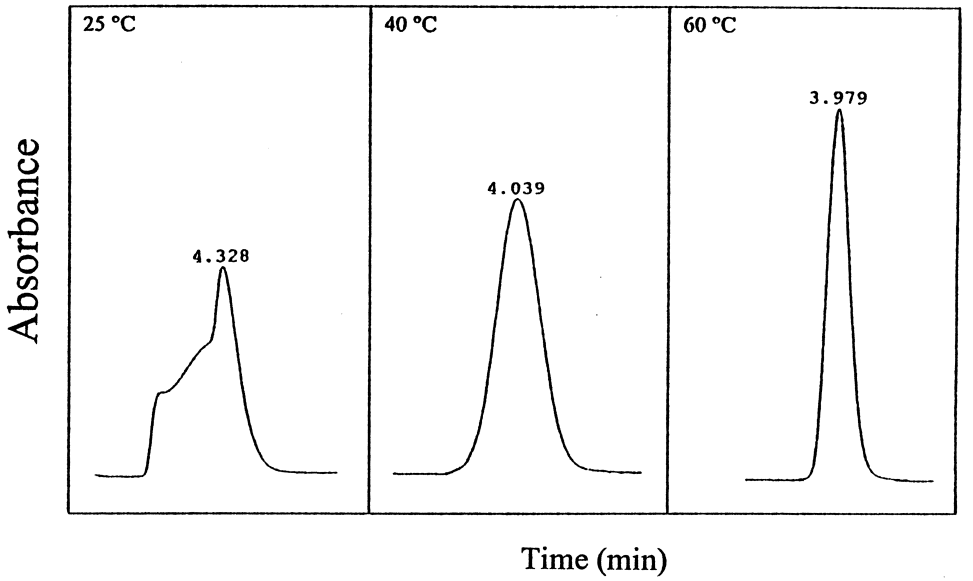

Fig. 2. Effect of column temperature on the peak shape and retention of enalapril. Mobile phase: phosphate buffer, pH 2–acetonitrile (60:40, v/v); flow-rate: 1.0 ml/min; stationary phase Supelco LC 18, 5 μm (250×4.6 mm I.D.).

3.3. Effect of pH

The influence of the phosphate buffer pH on the peak shape, retention times and the separation selectivity was investigated in the range 2–6 using a mobile phase consisting of 20 m*M* phosphate buffer–acetonitrile (70/30: v/v) at ambient temperature. The flow-rate was 2 ml/min.

As can be seen in Fig. 3, two distinct peaks were obtained at the different fixed buffer pH indicating the presence of the *cis* and *trans* forms. In addition the increase of pH led to the decrease of the retention times of both isomers. This result is in agreement with similar studies reporting that the retention times of zwitterionic form of amino acids or peptides is lower than that of corresponding cationic form [10,15,20]. However this finding is different to that reported for enalapril when a bonded C_8 phase was used and was explained by a predominant cation-exchange process due to the presence of unreacted silanol groups [19]. On the other hand, separation selectivity is improved with increasing pH (Fig. 4) but no baseline separation was obtained showing that a significant fraction of the substance was present in the reaction zone between the two peaks. Nevertheless, this observation indicates that the two isomers have different acidities and therefore a different pK_a. Such difference in acidity of *cis* and *trans* conformers has been reported for captopril, amino acids and peptides [2,21].

3.4. Influence of counter ion

Tetrabutylammonium hydrogensulfate (TBA) and sodium octanesulfonate (NaOS) were tested as ionic modifiers in order to investigate their effect on both peak shape and retention time.

They were added to mobile phases made of 20 m*M* phosphate buffer–acetonitrile (60:40, v/v) at ambient temperature. Since the pK_a values of enalapril are 2.97 (the carboxyl group) and 5.35 (the amine group) at 25°C [19], enalapril possesses a net positive charge at pH 2 and a net negative charge at pH 6.5. Thus the experiment performed with TBA (positive counter ion) was carried out with the mobile phase having an apparent pH adjusted to 6.5 while that done with NaOS (negative counter ion) is achieved at an apparent pH of 2.

As can be seen in Fig. 5, at lower TBA concentration an increase of the retention time was observed with a modification of the peak shape whereas at higher TBA concentration (100 m*M*), a

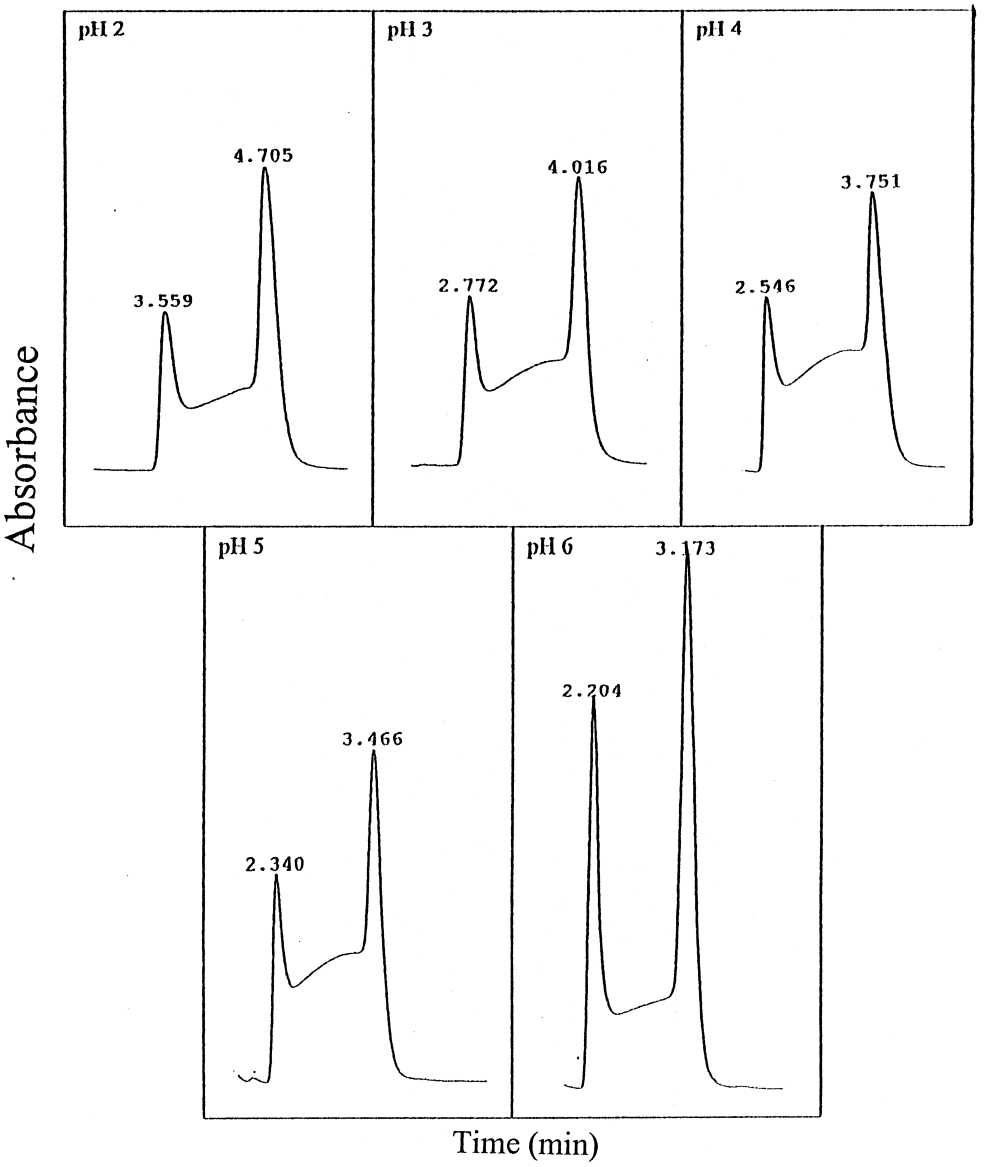

Fig. 3. Effect of pH on the peak shape and retention of enalapril. Mobile phase: phosphate buffer–acetonitrile (70:30, v/v); flow-rate: 2.0 ml/min; ambient temperature: stationary phase Supelco LC 18, 5 μm (250×4.6 mm I.D.).

single peak with a lower retention time was obtained which probably arises from the incipient micelle formation coupled with ion-pair formation in the mobile phase [22].

Results obtained with NaOS are shown in Fig. 6. With increasing concentration of this counter ion in the mobile phase, a more pronounced bimodal peak with a high retention time was obtained corresponding to the usual behaviour observed for an ion pairing system. In this way 20 m*M* NaOS was used at pH 7 to improve rotamer separation of similar product at low temperature [17] whereas 20 m*M* sodium heptanesulfonate (pH 2.5) with an acetonitrile–THF mixture was used for the elution and the separation of five ACE inhibitors [2].

However, another study reported that the addition

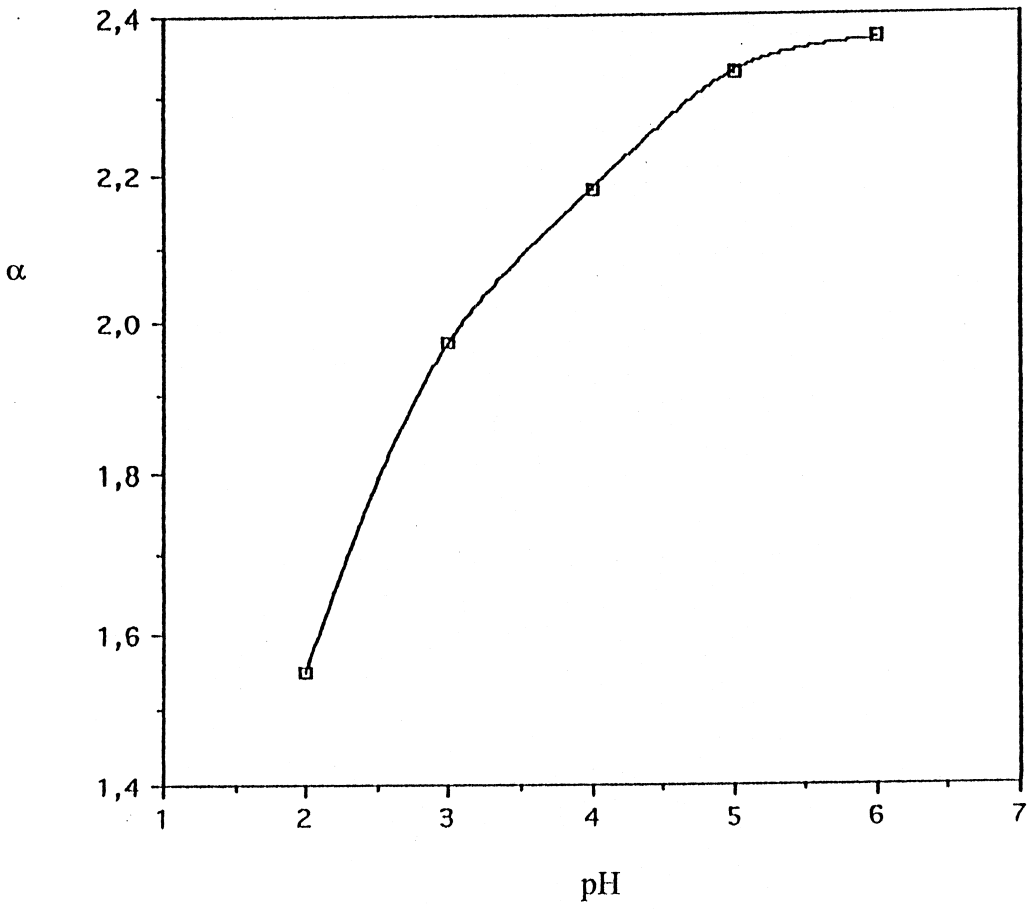

Fig. 4. Influence of the pH on the selectivity of enapril isomers. Conditions as in Fig. 3.

of sodium dodecylsulfate or the cetyltrimethylammonium bromide to the mobile phase (methanol–phosphate buffer) at a flow-rate of 0.5 ml/min led to a single peak of enalapril at ambient temperature [10]. A comparison between these findings cannot be considered since differences in operating conditions (pH, organic modifier, temperature, flow-rate) between these studies were noted. Nevertheless, these results indicate that by controlling experimental conditions, the addition of a counter ion can either improve the rotamer separation or allow the elimination of the peak splitting of such products.

3.5. Influence of the uncharged modifier

Acetonitrile, methanol and THF were tested as uncharged modifiers in aqueous phosphate buffer pH 2 at ambient temperature. The amounts of the organic modifiers were adjusted in order to obtain a comparable retention times. Their influence on the peak shape can be seen in Fig. 7. Using THF as organic modifier, the elution order of the conformers was reversed compared with acetonitrile. Similar observations about reversals of elution order caused by changes of organic modifiers on RPLC were previously reported for ramipril [3]. In addition, the use of methanol as organic modifier had a great influence on the elution profile since it led to a single peak even at lower concentration (40%) and ambient temperature giving an elution time of enalapril at 23.6 min. In this way, it was reported that the use of acetonitrile instead of methanol as the non-polar

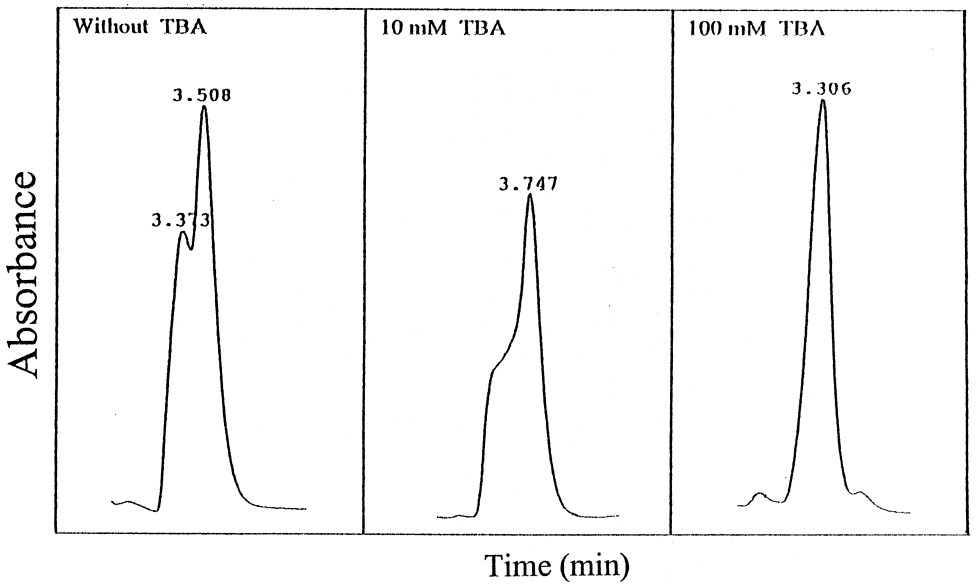

Fig. 5. Influence of tetrabutylammonium hydrogensulfate on the peak shape and retention time of enalapril; mobile phase (apparent pH 6.5): phosphate buffer, pH 6.5–acetonitrile (60:40, v/v); flow-rate: 1.0 ml/min; stationary phase: Supelco LC 18, 5 μm (250×4.6 mm I.D.).

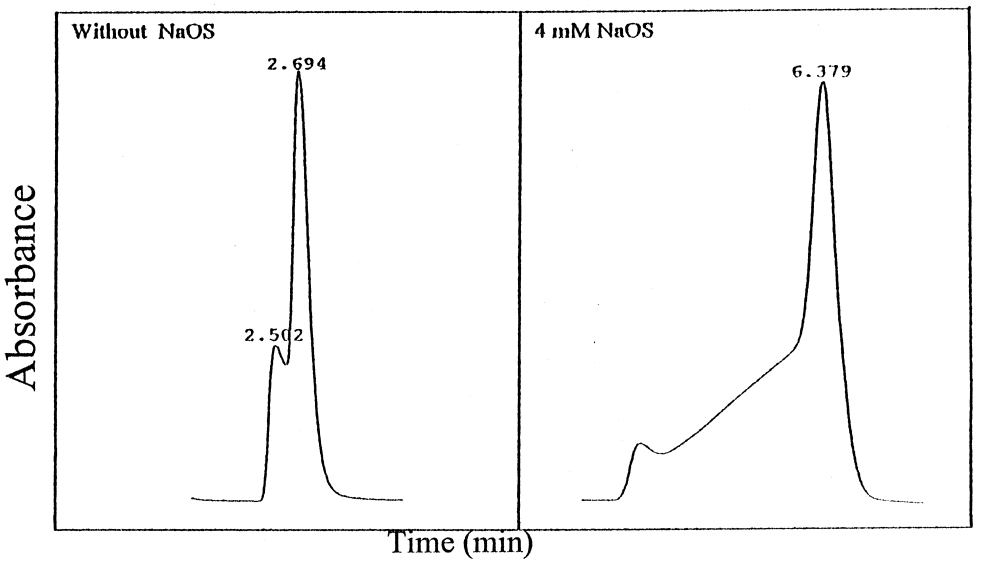

Fig. 6. Influence of sodium octanesulfonate on the peak shape and retention time of enalapril; mobile phase (apparent pH 2): phosphate buffer, pH 2–acetonitrile (60:40, v/v); ambient temperature: flow-rate 2.0 ml/min. stationary phase Supelco LC 18, 5 μm (250×4.6 mm I.D.).

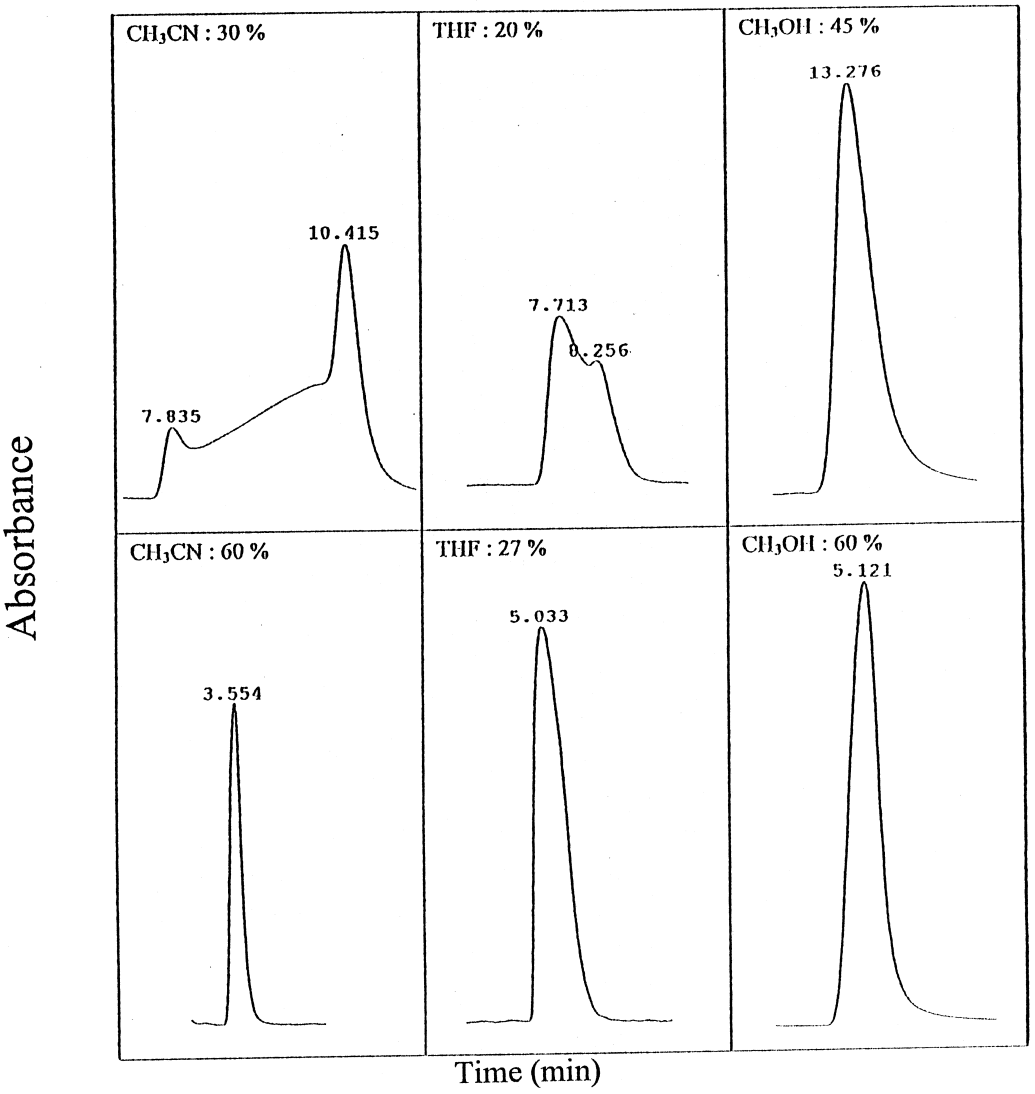

Fig. 7. Effect of organic modifier on the peak shape and retention time of enalapril; mobile phase: phosphate buffer, pH 2–organic modifier (concentrations are indicated); flow-rate 1.0 ml/min; ambient temperature: stationary phase Supelco LC 18, 5 μm (250×4.6 mm I.D.).

component of the mobile phase improved the rotamer separation of similar products [17]. On the other hand, as could be expected in reversed-phase systems, an increase in the organic modifier concentration resulted in a decrease in retention time. Moreover the elution of enalapril as a single peak was obtained.

However, it appears that acetonitrile or THF can be indicated for rotamer separation while methanol is preferable for eluting enalapril as a single peak at the laboratory temperature. Thus, a mobile phase consisting of 20 m*M* phosphate buffer, pH 2–methanol (40:60) at a flow-rate of 1 ml/min and at ambient temperature was applied for the elution and the separation of a mixture of enalapril and its degradates: diacid and diketopiperazine. The obtained chromatogram (Fig. 8) shows that all compounds are well separated in <10 min. This result indicates that

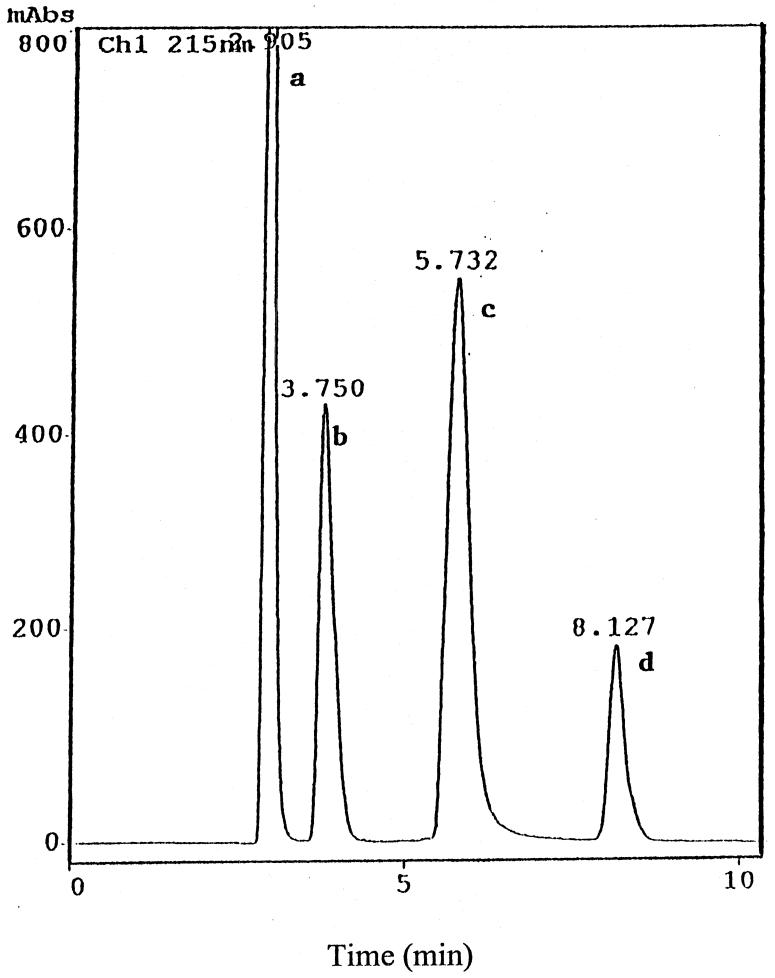

Fig. 8. Chromatograms showing the separation of maleic acid (a) diacid (b) enalapril (c) and diketopiperazine (d); mobile phase: phosphate buffer, pH 2–methanol (40:60, v/v); flow-rate: 1.0 ml/min; ambient temperature: stationary phase: Supelco LC 18, 5 μm (250×4.6 mm I.D.). Enalapril maleate and diacid: 0.1 mg/ml and diketopiperazine: 0.2 mg/ml.

methanol proved to be a useful organic modifier able to provide a single symmetric peak for enalapril with a good resolution at ambient temperature.

3.6. NMR study

The ^{1}H NMR spectrum of enalapril maleate is shown in Fig. 9. In the 3.4–4 ppm region, the spectrum exhibits two distinct sets of signals for each type of proton, which indicates the existence of two contributing isomers. These rotamers are assigned to the *cis–trans* equilibrium of the rotation around the amide bond. The isomer ratio was integrated to be 70:30. A similar results were obtained in a previous NMR studies of others proline containing substances [2,3].

4. Conclusion

Peak shape and retention time of enalapril are greatly affected by operating conditions. However elution of enalapril as a single peak and its separation from its degradates can be achieved at ambient

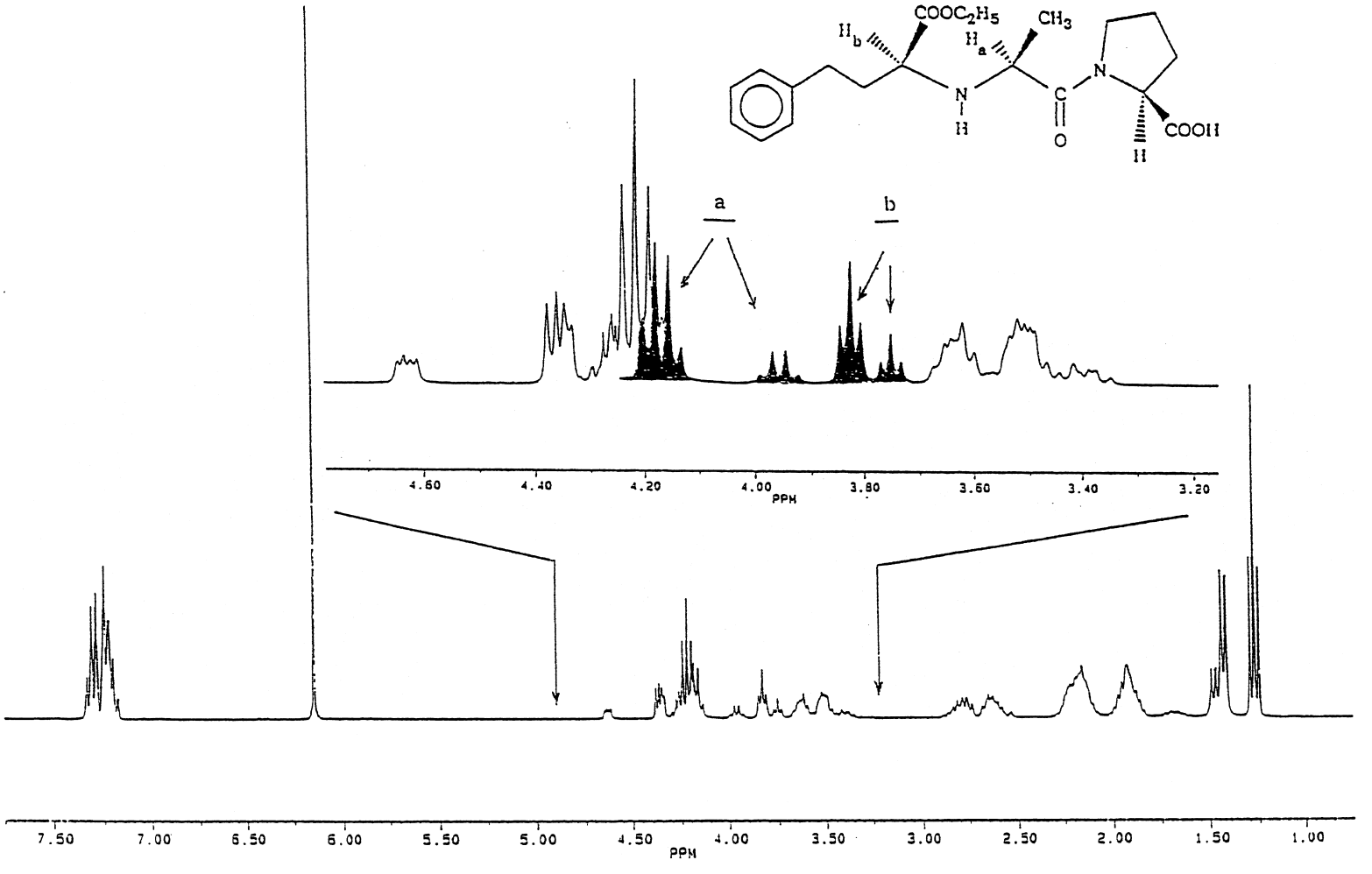

Fig. 9. ^{1}H NMR spectrum of enalapril maleate in [$^{2}H_{6}$ dimethylsulfoxide].

temperature by the appropriate choosing of organic modifier and pH. On the other hand, negatively charged counter ion can be used for further improvement of rotamer separation method.

References

[1] G. Simon, S. Morioka, D.K. Snyder, J.N. Cohon, Clin. Pharmacol. Ther. 34 (1983) 459.
[2] D. Bonazzi, R. Gotti, V. Andrisano, V. Carvini, J. Pharm. Biomed. Anal. 16 (1997) 431.
[3] S. Gustafsson, B-M. Eriksson, I. Nilsson, J. Chromatogr. 506 (1990) 75.
[4] D.L. Rabenstein, A.A. Isab, Anal. Chem. 54 (1982) 526.
[5] A. Skogolf, I. Nilsson, S. Gustafsson, J. Deinum, P.O. Gothe, J. Biochem. Biophys. 104 (1990) 22.
[6] J.F. Brandts, H.R. Halvorson, M. Brennan, Biochemistry 14 (1975) 4953.
[7] X.Z. Qin, D.S.T. Nguyen, P.D. Ip, J. Liq. Chromatogr. 16 (1993) 3713.
[8] J. Jacobson, W. Melander, G. Vaisnys, C. Horvath, J. Phys. Chem. 88 (1984) 4536.
[9] S. Frieb, B. Hartrodt, K. Neubert, G.J. Krauss, J. Chromatogr. 661 (1994) 7.
[10] J. Salamoun, K. Slais, J. Chromatogr. 537 (1991) 249.
[11] B.R. Thomas, S. Ghodbane, J. Liq. Chromatogr. 16 (1983) 2006.
[12] X-Z. Qin, P.D. Ip, W.E. Tsai, J. Chromatogr. 626 (1992) 251.
[13] D.E. Henderson, C. Horvath, J. Chromatogr. 368 (1986) 303.
[14] R. Hana, A. Wada, J. Chromatogr. 394 (1987) 273.
[15] W.R. Melander, J. Jacobson, C. Horvath, J. Chromatogr. 234 (1982) 269.
[16] A. Kalman, F. Thunecke, R. Schmidt, P.W. Schiller, C. Horvath, J. Chromatogr. 729 (1996) 155.
[17] D.E. Henderson, J.A. Mello, J. Chromatogr. 499 (1990) 79.
[18] P.D. Ip, S.G. Brenner, J.M. Stevenson, S. Lindenbaum, A.W. Douglas, S. Klein, J.A. Mccauley, J. Pharmaceutics 28 (1986) 183.
[19] X-Z. Qin, J. DeMacro, D.P. Ip, J. Chromatogr. 707 (1995) 245.
[20] E.P. Kroeff, D.T. Pietrzyk, Anal. Chem. 50 (1978) 502.
[21] C.A. Evans, D.L. Rabenstein, J. Am. Chem. Soc. 96 (1974) 7312.
[22] J.H. Knox, R.A. Hartwick, J. Chromatogr. 204 (1981) 3.