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RP-HPLC and NMR study of *cis–trans* isomerization of enalaprilat

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Abstract

The angiotensin converting enzyme inhibitor, enalaprilat can exist in solution as *cis* and *trans* conformers which interconvert around the amide bond at room temperature. A HPLC with UV detection was performed to study the influence of various chromatographic operational conditions on both rotamers separation and elution of enalaprilat as a single peak. In addition nuclear overhauser enhancement difference was used for the identification of the conformers. The isomer ratio integrated from the obtained ^{1}H NMR result were 71.5:28.5 and 76:24 at 298 and 279 K, respectively where the *trans* was the major form.

Keywords: Enalaprilat; Isomer separation; Liquid chromatography; NMR

1. Introduction

Enalaprilat, (*S*)-1-[*N*-(1-carboxy-3-phenylpropyl)-L-alanyl]-L-proline dihydrate (Fig. 1), an inhibitor of the angiotensin converting enzyme [1] has been marketed in injectable dosage form to control hypertension [2].

In solution, enalaprilat can exist as *trans* and *cis* since it is a dipeptide containing L-proline. HPLC and capillary electrophoresis have proven to be a meaningful tools for conformational and kinetic studies of such products [2–9]. On the other hand, the HPLC methods developed for enalaprilat allow its elution as a single sharp peak at lower buffer pH by using high temperature [10,11] or with appropriate amount of organic modifier [12,13]. But so far, no studies have described the influence of the flow rate, nature of organic solvent and counter-ions. In addition the separation and the identification of the rotamers is still lacking.

However, we described in a recent paper [12] that RP-HPLC allows the study of the isomerization of enalapril, the enalaprilat prodrug (Fig. 1), as a function of chromatographic conditions. We also demonstrated that HPLC enables both the

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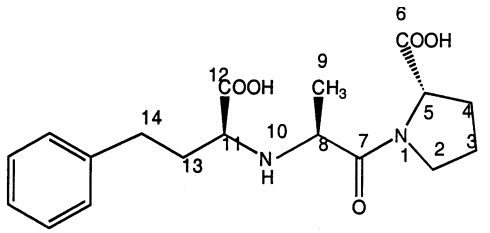

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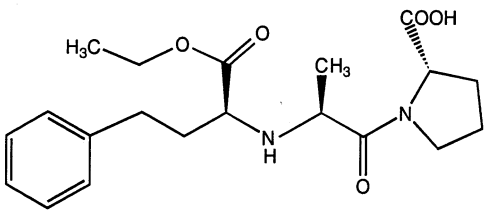

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Fig. 1. Chemical structure of: (1) enalaprilat; (2) enalapril.

elution of enalapril as a single peak and its separation from its impurities by using more practical experimental conditions.

The goal of the present work is to shed further light on the influence of the flow rate, temperature, pH, organic solvent and counter-ions on the retention time, peak shape and resolution of the two isomers of enalaprilat. Accordingly, the optimal chromatographic conditions for separation of the rotamers were consequently used in performing NMR study in order to identify the *cis* and *trans* forms and to define their ratio.

2. Experimental

2.1. Chromatography

Liquid chromatographic analyses were performed using a LC-10AT vp pump (Shimadzu) connected to an UV–Vis detector (SPD-10A vp, Shimadzu) set at 215 nm. Injection was performed using an auto injector (SIL-10AD vp). The data were collected on a Dell optiles GX110 computer. The whole chromatographic system was controlled by the same computer, using Shimadzu Class-vp 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-10AC vp Shimadzu) was incorporated into the system. The pH of mobile phase buffers was adjusted by mean of a model CG 825 pH meter from Schott (Germany).

2.2. Nuclear magnetic resonance (NMR)

^{1}H NMR spectra and nuclear overhauser enhancement difference (NOEDIFF) spectra were obtained at 300.13 MHz on Bruker spectrometer. The probe temperature was 298 and 279 K. ^{1}H chemicals shifts were measured relative to tetramethylsilane [TMS, $(CH_3)_4Si$]. The spectrum width spectra was 4201.68 Hz and relaxation time was 0.5 s.

Semi empirical calculations were supported by the GAUSSIAN-98 algorithm using the AM1 Hamiltonian [14].

2.3. Sample, reagents and mobile phase

Enalaprilat, (*S*)-1-[*N*-(1-carboxy-3-phenylpropyl)-L-alanyl]-L-proline dihydrate was kindly provided 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). A sample solution was prepared by dissolving an amount of enalaprilat in water to yield the concentration of 1 mg/ml. This preparation was stored at 4 °C for a maximum of 1 month. The mobile phase consisted of 20 mM phosphate buffer and an organic modifier (acetonitrile, methanol or THF) to which a counter-ion was added when it is necessary. 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 sample solution, of enalaprilat was injected.

For NMR study, a sample solution was prepared by dissolving an appropriate amount of enalaprilat in 1 ml of mixture of D_2O/CD_3CN (9/1) to obtain the desired concentration of enalaprilat (0.2 mol/l).

3. Results and discussion

3.1. Flow rate

At flow rates of 0.3, 1, 1.5 and 2 ml/min, the effect of the time scale of HPLC elution in the column on the peak shape of enalaprilat was investigated at 25 °C (Fig. 2). The mobile phase was a mixture of 20 mM phosphate buffer pH 7–acetonitrile (90/10, v/v). Fig. 2 shows that higher flow rate yielded a peak corresponding to the two isomers while the lower flow rate of 0.3 ml/min produced, a single peak ($t_R = 14.3$ min). This effect of the flow rate on the peak shape has been reported for enalapril [12] and similar compounds having relaxation times of *cis–trans* interconversion commensurate to their retention times [13,15–18].

3.2. Column temperature

The chromatographic behavior of enalaprilat obtained at several column temperatures with a mobile phase consisting of a mixture of 20 mM phosphate buffer pH 7–acetonitrile (90/10, v/v) at a flow rate of 2 ml/min is shown in Fig. 3. Elevated temperature led to a deterioration of separation of the two conformers. Moreover, at 55 °C enalaprilat was eluted as a narrow single peak probably due to the high isomerization rate of the tow conformers. On the other hand, at low temperature (6 °C) the two isomers were resolved almost completely indicating that the interconversion rate was slowed down. From the given chromatograms the isomer ratio was integrated to be 75:25. This result is similar to those reported earlier, demonstrating that high temperature was useful for elution of proline-containing substances as a single peak [9,12,13,15,19], whereas a low temperature had a potential effect on the separation of isomers [16,18,20–22].

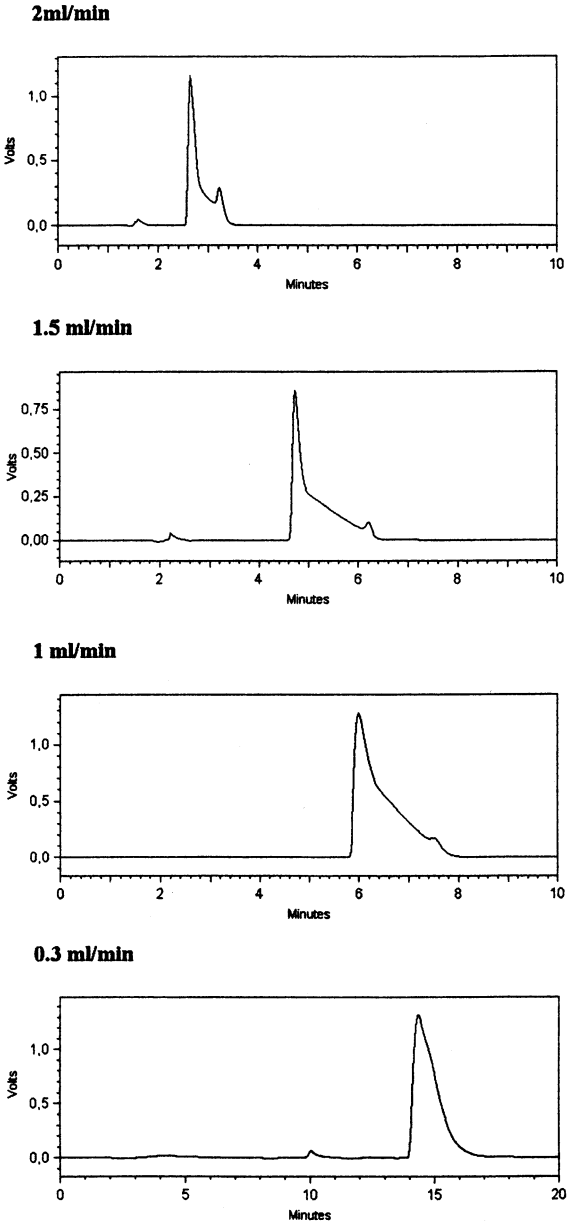

Fig. 2. Effect of flow rate on the peak shape of enalaprilat; mobile phase: phosphate buffer, pH 7–acetontrile (90:10, v/v); temperature 25 °C; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

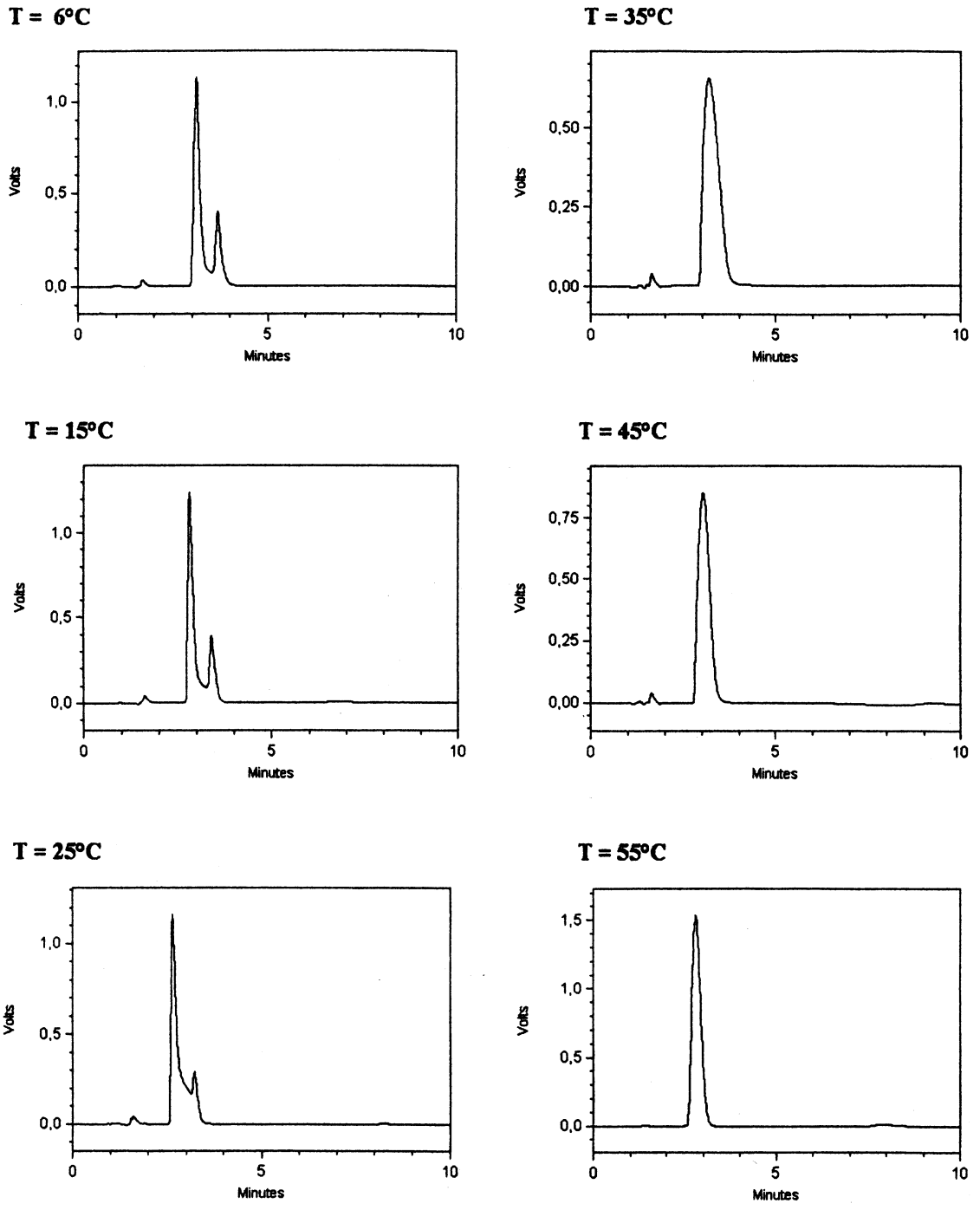

Fig. 3. Effect of column temperature on the peak shape and retention time of enalaprilat; mobile phase: phosphate buffer, pH 7–acetontrile (90:10, v/v); flow rate: 2.0 ml/min; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

However, the separation of the two conformers suggests that their hydrophobic surface areas are different as it can be seen from the molecular structures of *cis* and *trans* enalaprilat in Fig. 4. Indeed, the *trans* form has a lower hydrophobic surface area than the *cis* where its polar and non-polar groups are separated with respect to the plane through the two α carbons. Thus, the *trans* broading to the hydrocarbonaceous ligates of the stationary phase is less strong than that of the *cis* conformer and therefore it can be expected that the retention of the *cis* form is greater than the one of the *trans* isomer. This is argued by earlier studies on the separation of L-alanyl-L-proline isomers [13,15] and others conformers of peptides containing proline [20].

3.3. Influence of the uncharged modifier

To investigate the effect of the nature and the amount of the organic modifier, acetonitrile, methanol and THF were used in aqueous phosphate buffer pH 2 at 25 °C. The concentration of the organic solvents were adjusted in order to obtain comparable retention times. The obtained chromatograms are depicted in Fig. 5.

The three organic solvents had the same influence on the peak shape of enalaprilat. At lower concentration a comparable chromatogram profile showing a bimodal peak was obtained with the three solvents and therefore indicates in this case that both hydrophobic and polar group selectivity do not change with the different phase systems [23]. This result is different from those obtained for enalapril and ramipril where THF inverted the order elution of their isomers compared with acetonitrile [12,20]. In addition enalapril was always eluted as a single peak using methanol as organic modifier even at low concentration [12].

However, increase of the amount of organic modifier led to significant improvement in the peak shape of enalaprilat and higher concentration of the organic solvent yielded a single peak with a decrease of the retention time. This result is consistent with those reported for similar products [9,11,12,20].

3.4. Effect of pH

As was mentioned earlier, the pH of mobile phase influences both peak shape and retention time of proline containing substances. The chromatograms were obtained with a mobile phase mixture of 20 mM phosphate buffer pH in the range 3–7 and acetonitrile (90/10, v/v) at 25 °C and a flow rate of 2 ml/min.

At higher pH, two distinct peaks without baseline resolution corresponding to the two isomers were observed. On the other hand, it clearly appears that the lower the pH, the higher is the retention time. Indeed, at pH 3 enalaprilat possesses a net positive charge since its carboxyl groups have a pK_a value between 2 and 3, and its amine group has a pK_a value between 5 and 6 [24,25]. This positively charged enalaprilat can interact with unreacted silanols and therefore increase of t_R is obtained. This result, conform to the explanation that protonated amines are sensitive to unreacted silanols [23], supports the earlier findings that electrostatic interaction of enalaprilat and similar products with surface silanols occurs at low pH and therefore increases their retention times [11,25]. Moreover, it was reported that the silanophilic interaction with

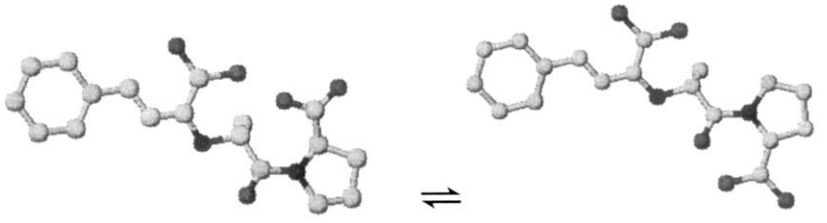

Fig. 4. Perspective molecular structures of *cis* and *trans* form of enalaprilat.

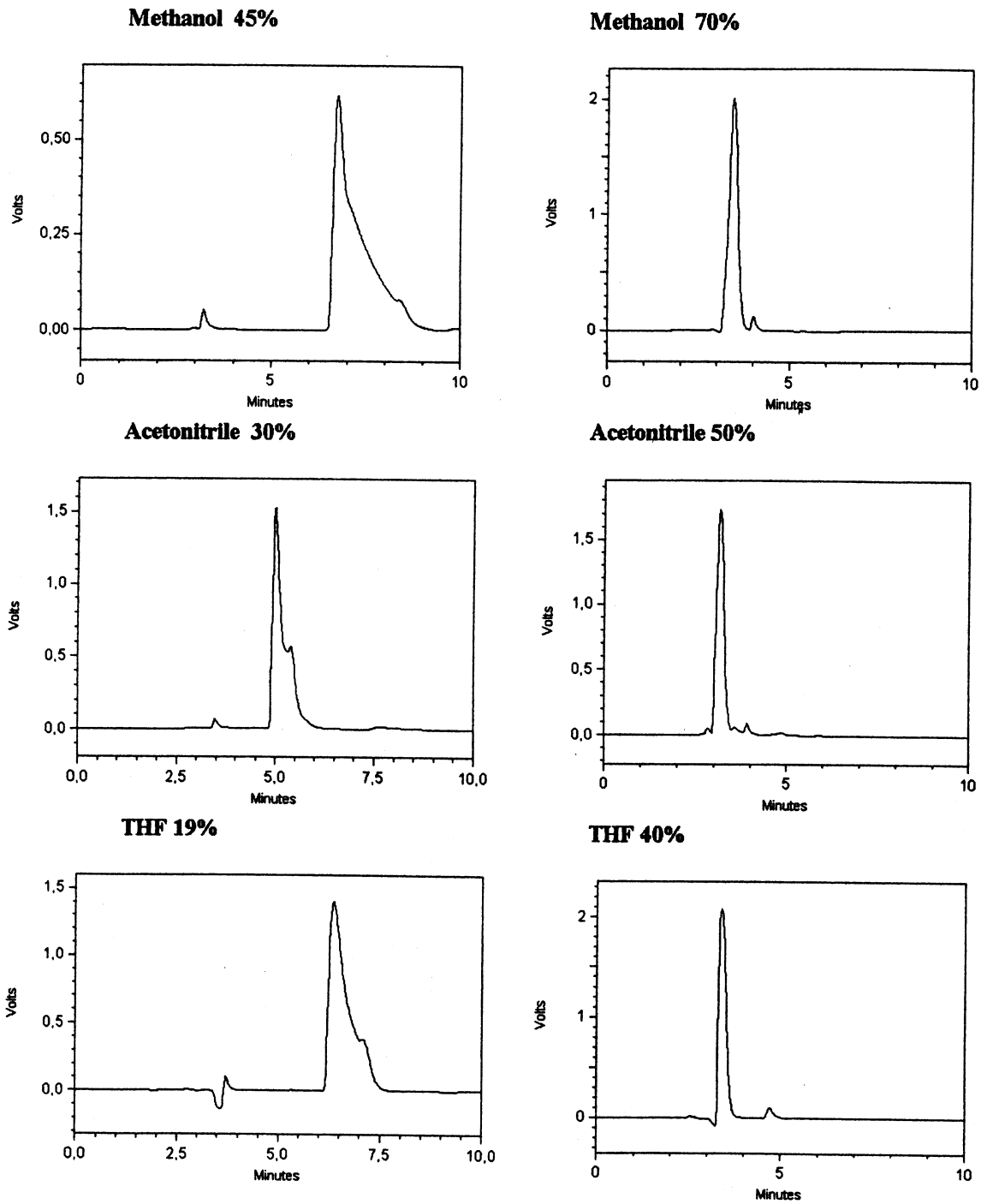

Fig. 5. Effect of organic modifier on the peak shape and retention time of enalaprilat; mobile phase: phosphate buffer, pH 2–organic modifier (concentrations are indicated); flow rate 1.0 ml/min; temperature 25 °C; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

ammonium ion appears to be predominant in the retention of these species [23] and peaks of such eluites are often broad and asymmetric [25]. Besides, at low pH, a monodisperse peak of enalaprilat with excessive broading was obtained as shown in Fig. 6.

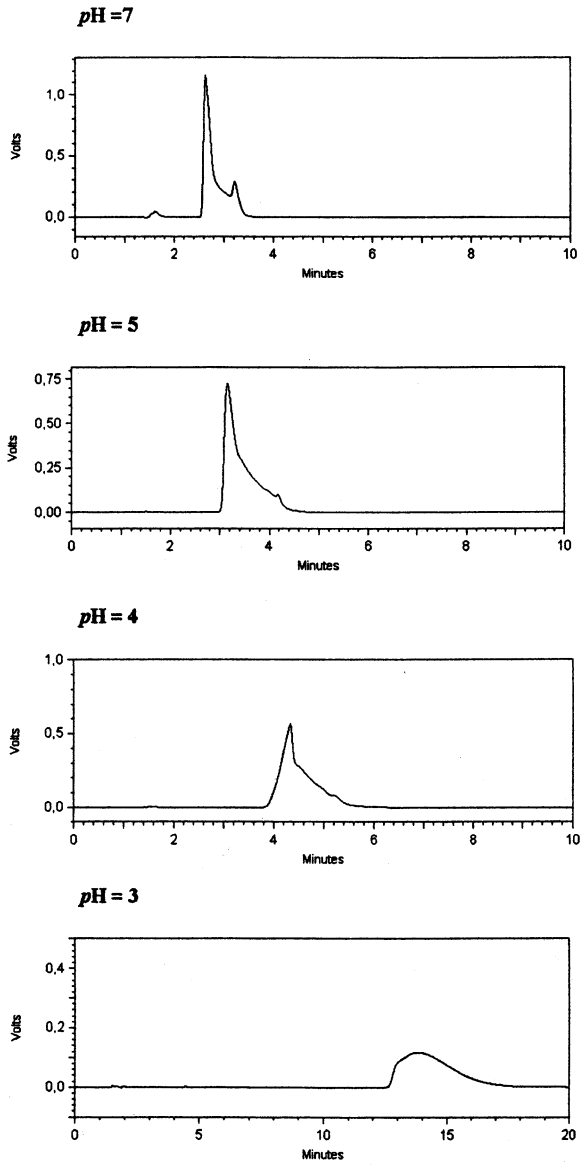

Fig. 6. Effect of pH on the peak shape and retention time of enalaprilat; mobile phase: phosphate buffer–acetontrile (90:10, v/v); flow rate: 2.0 ml/min; temperature 25 °C; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

3.5. Influence of counter-ion

Both tetrabutylammonium hydrogenosulfate (TBA) and sodium octanesulfonate (NaOS) at different amounts were used as ionic modifiers. They were separately added to mobile phases made of 20 mM phosphate buffer–acetonitrile at 25 °C. The proportions of buffer and organic solvent in the eluent are (90/10, v/v) and (70/30, v/v) for TBA and NaOS, respectively. With respect to the pK_a values of enalaprilat and in order to obtain ion-pair formation between the eluite and the pairing agents, apparent pH of mobile phases were adjusted to 7 and 2 for experiments with TBA and NaOS, respectively. The effect of these two counter-ions on both peak shape and retention time is illustrated in Figs. 7 and 8.

The increase of the concentration of the two pairing agents led to an increase of the retention time of enalaprilat, which was attributed to the well known effect of the counter-ions at low concentrations [26]. Moreover, a single symmetrical peak was obtained with either TBA or NaOS.

However, at higher TBA concentration (100 mM), a decrease of the retention time was observed which, arises from the incipient micelle formation couples with ion-pair formation in the mobile phase [26]. This effect of TBA on the elution profile of enalaprilat is comparable to the one reported for enalapril in previous studies [9,12].

On the other hand, the result obtained with NaOS is different to those observed for others proline-containing substances where this counter-ion improves rotamer separation [12,21]. Nevertheless, it can be stated that pairing-agent is a significant parameter for both control of the retention time and elution of enalaprilat as a single peak even at ambient temperature.

3.6. NMR study

The ^{1}H NMR spectrum of enalaprilat in D_2O/CD_3CN (9/1, v/v) is shown in Fig. 9. In the 1–4.2 ppm region, the spectrum exhibits two distinct sets of signals for each type of proton ($\Delta\delta = 0.2$ ppm) confirming thus the existence of the two conformers. These isomers are assigned to a *cis–trans*

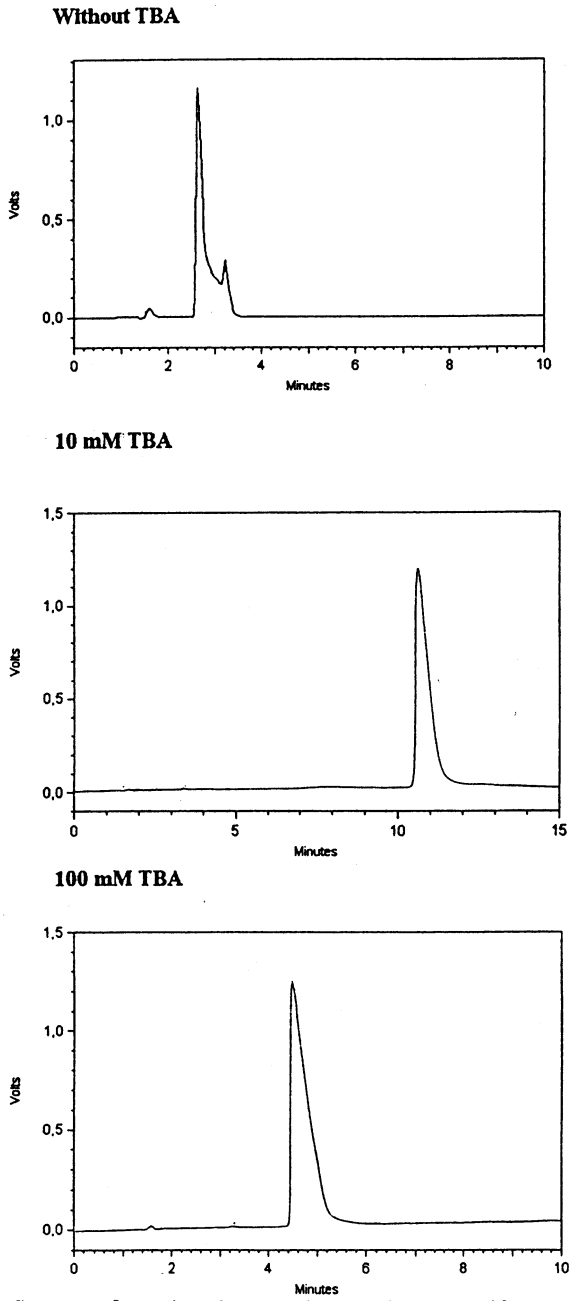

Fig. 7. Influence of TBA on peak shape and retention time of enalaprilat; mobile phase (apparent pH 7): phosphate buffer, pH 7–acetontrile (90:10, v/v); flow rate: 2.0 ml/min; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

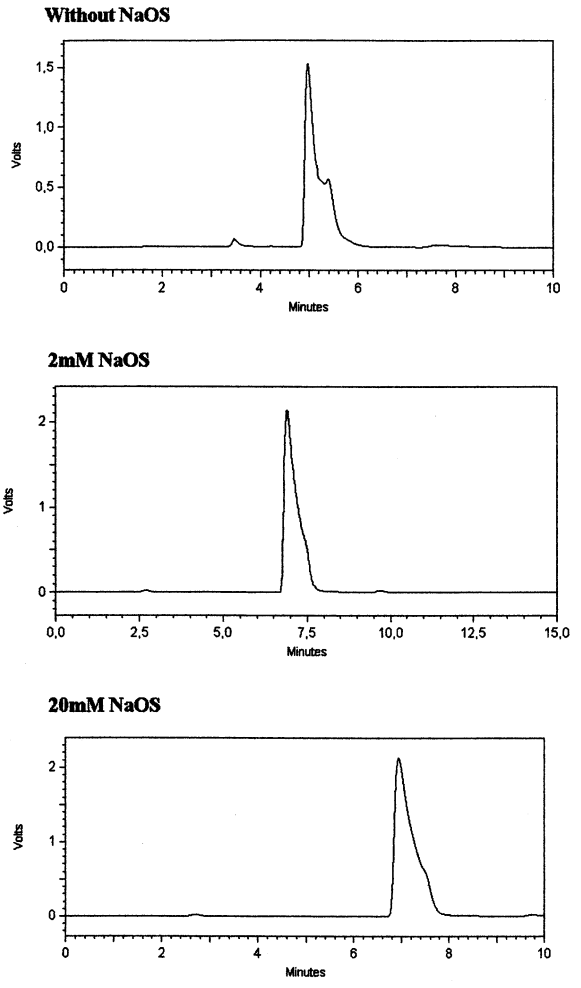

Fig. 8. Influence of sodium octanesulfonate on peak shape and retention time of enalaprilat; mobile phase (apparent pH 2): phosphate buffer, pH 2–acetontrile (70:30, v/v); flow rate: 2.0 ml/min; stationary phase: Supelco LC 18, 5 μm (250 × 4.6 mm I.D.).

equilibrium of the rotation around the amide bond. It is worth noting that this equilibrium appears to be slow compared to the time scale of NMR [27,28].

Using the area of resonance signals of methyl groups (1.3–1.4 ppm), the isomer ratio was integrated to be 71.5:28.5 and 76.9:23.1 at 298 and 279 K, respectively. The result obtained at 279 K is comparable to the one already determined by HPLC at the same temperature. We conclude that the major conformer in the ^{1}H NMR spectrum of

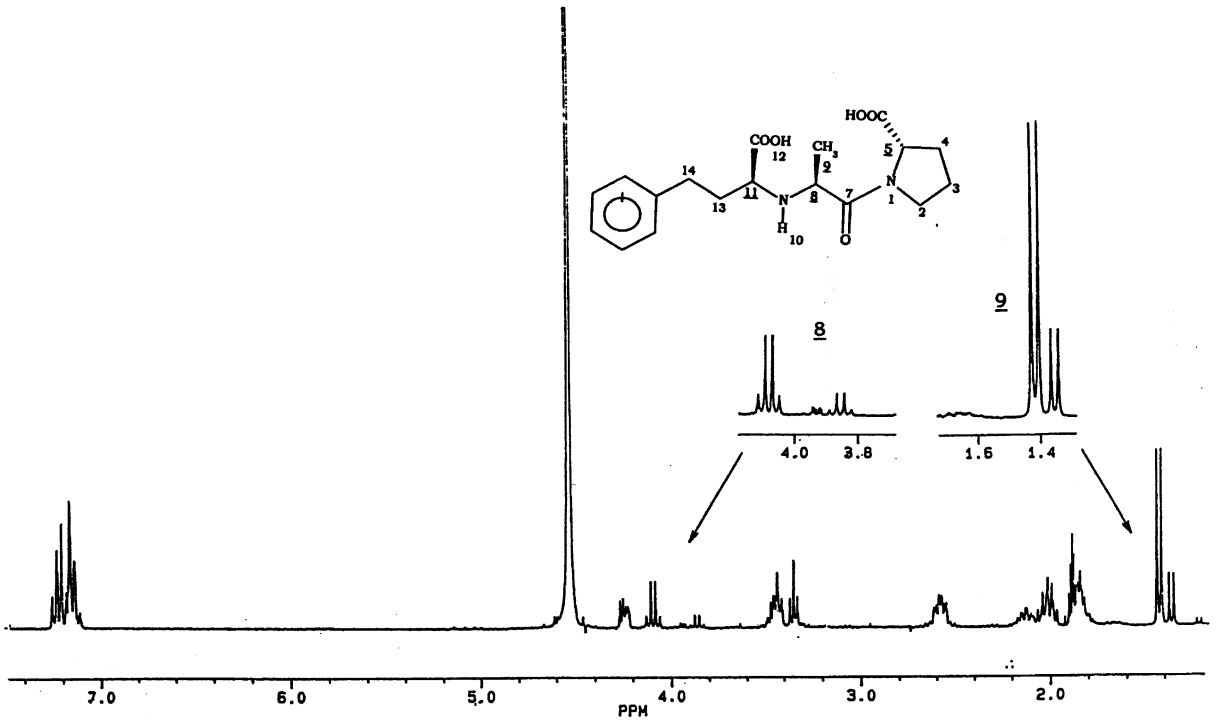

Fig. 9. ^{1}H NMR spectrum of enalaprilate in D_2O/CD_3CN (9/1, v/v).

enalaprilat corresponds to the first eluted peak in the HPLC chromatogram, which exists at the higher proportion. A similar study demonstrated this correspondence in the case of ramiprilat prepared in different solvents [29].

Based on the molecular structure of enalaprilat (Fig. 9), the distance between the nuclei in both conformers (*cis* and *trans*) was compared using a minimization algorithms to create low energy conformers of the two isomers, the distance between the nuclei in both conformers (*cis* and *trans*) was compared. It is revealed that in the *cis* conformer, H(5) is close to H(9) (2.1583 Å), while H(5) is at a much greater distance from H(11) (4.9308 Å). On the other hand, in the *trans* conformer, H(5) is further away from H(9) (4.7470 Å), while H(5) is at much greater distance from H(11) (6.8135 Å).

However, it is well known that the NOE effect (ξ) between two dipole–dipole interacting nuclei is inversely proportional to the distance $(r_{ij})^6$ between the irradiation site (i) and the measure one (j), respectively [30–32] according to the following formula:

$$\xi = f\left(\frac{1}{r_{ij}^6}\right).$$

Consequently, in the minor conformer, we expect a strong positive NOE ($\xi = 26$) at H(5) when H(9) is irradiated (Fig. 10a). Accordingly, in the major conformer we expect a low NOE ($\xi = 9$) at H(5) when H(9) is irradiated (Fig. 10b). In addition when H(11) is irradiated, we expect at H(5) a NOE ($\xi = 29$) in the minor conformer and ($\xi = 11$) in the major conformer (Fig. 10c and d). This implies that in the major conformer, the distance between the nuclei is higher than the one in the minor conformer.

These NOE results allowed to confirm that the major conformer in D_2O/CD_3CN, which is the first eluted peak in HPLC, corresponds to the *trans* conformer.

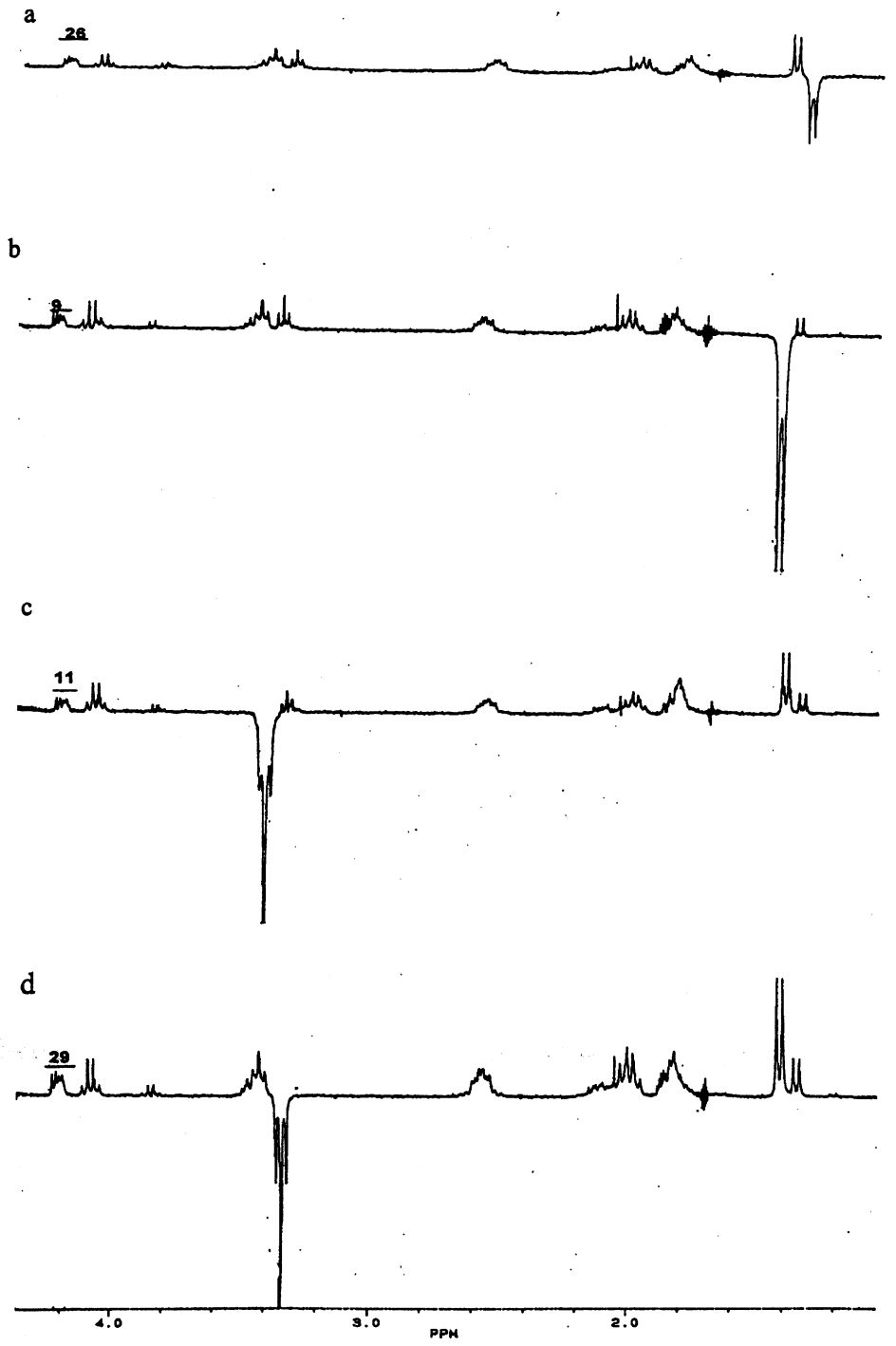

Fig. 10. NOE difference spectra of 20 mM enalaprilate in D_2O/CD_3CN, at 298 K and 300 MHz. Irradiation of H (9) and H (11): (a, c) minor conformer; (b, d) major conformer. The numbers next to the peaks represent the quantified NOE at H (5).

4. Conclusion

This study demonstrates that chromatographic conditions have a dramatic effect on peak shape and retention time of enalaprilat. It appears that elution of enalaprilat as a single sharp peak can be achieved at a temperature of 25 °C by using appropriate concentration of organic solvent together with a pH 2. However, low temperature is essential for the rotamer separation. On the other hand, the use of counter-ions improve peak shape with increasing the retention time of enalaprilat. In addition, this work shows that nuclear overhauser enhancement difference can be successfully applied to the identification of the two conformers.

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