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Effect of column temperature on the behaviour of some angiotensin converting enzyme inhibitors during high-performance liquid chromatographic analysis

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Abstract

The chromatographic behaviour of the ACE inhibitors lisinopril, enalapril and its two degradation products, enalaprilat (hydrolytic degradation product) and diketopiperazine (DKP) (cyclization degradation product) was studied as a function of column temperature and pH of the mobile phase. The rate of isomerization (which influences the peak shape or even peak splitting during chromatographic analysis) increases with temperature. The shape of the chromatographic peak for enalapril, enalaprilat and lisinopril is also pH dependent. At high temperature (80°C) and low pH (pH=2) all studied compounds appear on the chromatogram as a narrow chromatographic peak. Chromatographic peaks become broader or they split by lowering the column temperature. Enalapril appears at 6°C on the chromatogram in two peaks which belong to its *cis*- and *trans*-rotation isomers. Separation of the rotamers was confirmed by NMR spectroscopy. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Column temperature; Angiotensin-converting enzyme inhibitors; Lisinopril; Enalapril; Enalaprilat; Diketopiperazine

1. Introduction

Enalapril maleate (salt of enalapril and maleic acid 1:1) and lisinopril are two effective drugs for the treatment of renivascular hypertension and heart failure [1–3]. They act as inhibitors of the enzyme angiotensin convertase (ACE-inhibitor). It was observed that enalapril often contains as impurities the two degradation products enalaprilat (hydrolytic degradation product) and diketopiperazine (cyclization degradation product) [4]. The structures of all these compounds are shown in Fig. 1.

Enalapril is a dipeptide with a proline peptide bond. The configuration of the peptide bond can be either *cis* or *trans*. Although *trans*-configuration is preferential in peptides, *cis*-configuration is likely to occur in proline-containing peptides and *cis*–*trans* interconversion may appear due to the reduced barrier height. Rotation around the peptide bond is restricted due to the partial double bond character [5]. The double bond character can be decreased in solution at lower pH. The *cis*–*trans* interconversion

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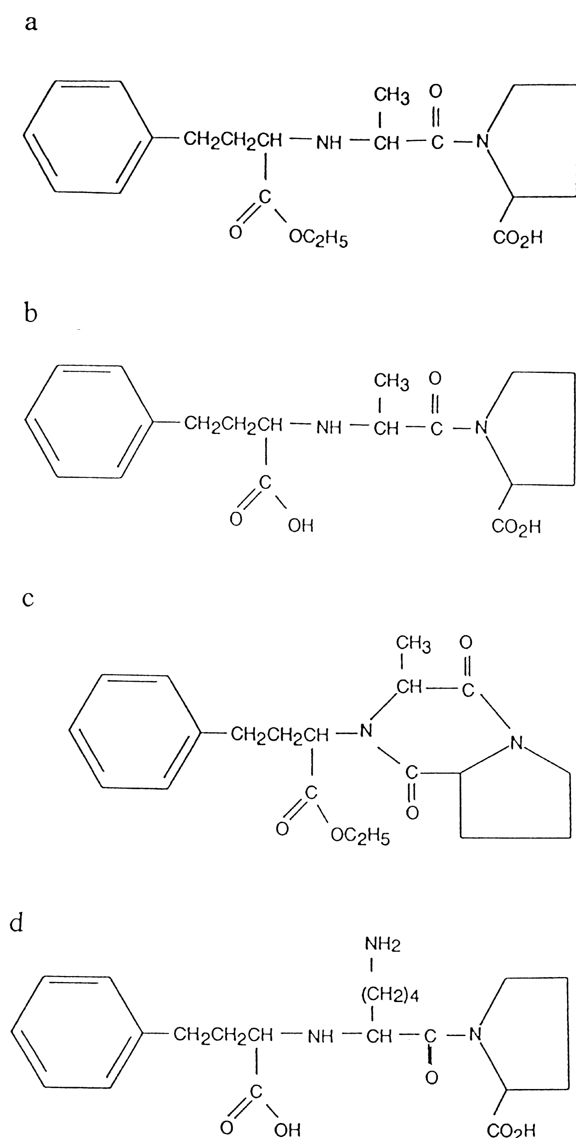
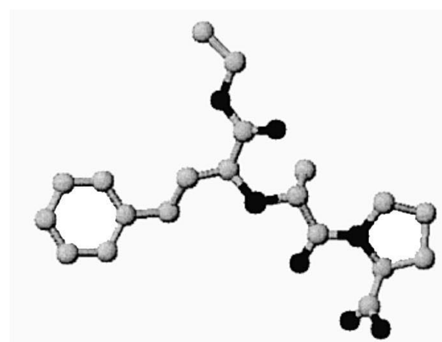
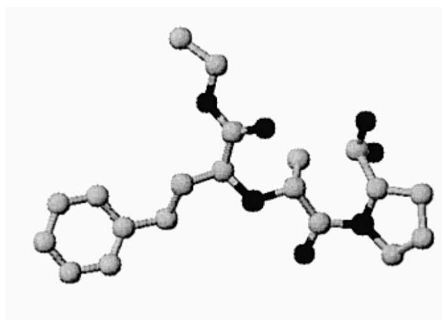


Fig. 1. Structures of enalapril (a), enalaprilat (b), enalapril-DKP (c) and lisinopril (d).

of enalapril in solution at room temperature, like that of other proline dipeptides, has a relaxation time of a few minutes [8,9]. Enalapril and related compounds are determined by high-performance liquid chromatography (HPLC). During chromatographic separations isomer interconversion also proceeds, which influences the peak shape or even causes peak splitting of ACE inhibitors. Peak splitting is usually undesirable in chromatographic analysis. It can lead to misinterpretation of the identity of a peak. Namely, it can be easily attributed to an impurity. Peak shape is strongly temperature, pH and flow-rate dependent due to slow the conformation of equilibrium proline containing peptides [6,7]. Gesquiere et al. reported several cases of medium sized synthetic peptides for which unusual chromatographic behaviour (unsymmetrical, split chromatographic peaks) were observed during reversed-phase HPLC at room temperature as the result of slow rotamer interconversion [7]. In contrast for all these compounds single sharp peaks were observed at a column temperature of 60°C. Also Melander et al. observed that dipeptides that contain proline may yield multiple peaks in reversed-phase HPLC [6]. Jacobson et al. pointed out that from the molecular structures of Ala-Pro *cis* and *trans* (Fig. 2) conformers, the *cis*



trans



cis

Fig. 2. Enalapril rotation isomers.

conformer has a larger hydrophobic surface area than the *trans* form and therefore its retention time is longer (using RP stationary phase) [9]. Various studies were reported where different chromatographic conditions were chosen for analysing several ACE-inhibitors aimed at achieving a symmetric, single chromatographic peak for each individual compound [4,10–13]. However, Xue-Zhi Qin et al. succeeded in separating the *cis*- and *trans*-rotamers of enalapril by use of micellar electrokinetic capillary chromatography in which 40–100 m*M* sodium dodecyl sulfate was added to the electrolyte buffer [14].

In our laboratory enalapril is routinely analysed by reversed-phase HPLC. At room temperature peak splitting can be readily observed for enalapril. We studied the effect of column temperature and pH on the elution profiles of enalapril. For comparative purposes, some other compounds (enalaprilat, enalapril-diketopiperazine and lisinopril) were also investigated in order to find similarities in the elution profiles.

The structures of isolated isomers were confirmed by NMR spectroscopy after complete separation of enalapril isomers on an analytical HPLC column.

2. Experimental

2.1. Reagents

Enalapril maleate, enalaprilat, diketopiperazine (DKP) and lisinopril were prepared in the Lek d.d. Pharmaceutical Company. Sodium dihidrogenphosphate-2-hydrate, sodium hydroxide and ortho-phosphoric acid (85%) were obtained from Riedel-de Haen (Seelze, Germany) and acetonitril LiChrosolv from Merck (Darmstadt, Germany). Deionized water of at least 18 Mohm was purified by an Elga UHQ (High Wycombe, Bucks, UK) apparatus.

2.2. Instrumental

HPLC analysis was performed with a Hewlett-Packard Series 1100 chromatograph (HP, Palo Alto, CA, USA). ACE inhibitors were separated on a 250 mm×4.6 mm I.D. chromatographic column packed with 5 μm Spherisorb Octyl (Waters Corporation, Milford, MA, USA). The column was in-house pressure-packed using a Knauer pump at a pressure of 600 bar.

The ACE inhibitors were eluted with 20 m*M* phosphate buffer at pH 2.0 and 7.0 containing various amounts of acetonitrile at different temperatures. Studied compounds have similar retention factors at selected chromatographic conditions, what makes them easier to compare. The flow-rate was set to 2 ml/min (except when isolating rotamers where rate was 1.8 ml/min). Twenty m*M* phosphate buffers were prepared using phosphoric acid or sodium hydroxide to adjust the pH to the desired values. Compounds were detected by a UV detector set to 215 nm. Standard solutions of individual ACE inhibitors (1 mg/ml) were prepared in the mobile phase and 20 μl of sample was injected.

^{1}H NMR measurements were performed on a 600 MHz instrument Varian INOVA 600 located at the NMR Centre, National Institute of Chemistry. The instrument was equipped with a 5 mm inverse detect probe IDpfg.

Perspective drawing of enalapril were prepared by using ACD/ChemSketch Version 4.55.

3. Results and discussion

3.1. Temperature effect

The purpose of observing the effect of column temperature on the peak shape of ACE-inhibitors was to determine the rate of isomerisation which influences their chromatographic peak shape. The effect of column and mobile phase temperature (20–80°C in 10°C steps) on the peak shape of enalapril is shown in Fig. 3. Separation on the same column was carried out using two mobile phases; an acidic phase (pH 2.0, Fig. 3a) and a neutral mobile phase (pH 7.0, Fig. 3b) containing in the first case 32% of acetonitrile and 17% of acetonitrile in the second. It can be seen from both Figs. (3a,b) that chromatographic peaks for enalapril become narrower at higher temperatures. Due to the higher rate of isomerization with increasing temperature the peak shape improves. A great difference in peak shape can be observed on comparing the chromatographic peak obtained by the mobile phases at pH 2.0 and 7.0. Namely, at pH 7.0 the C–N bond has a stronger double-bond character, which restricts rotation

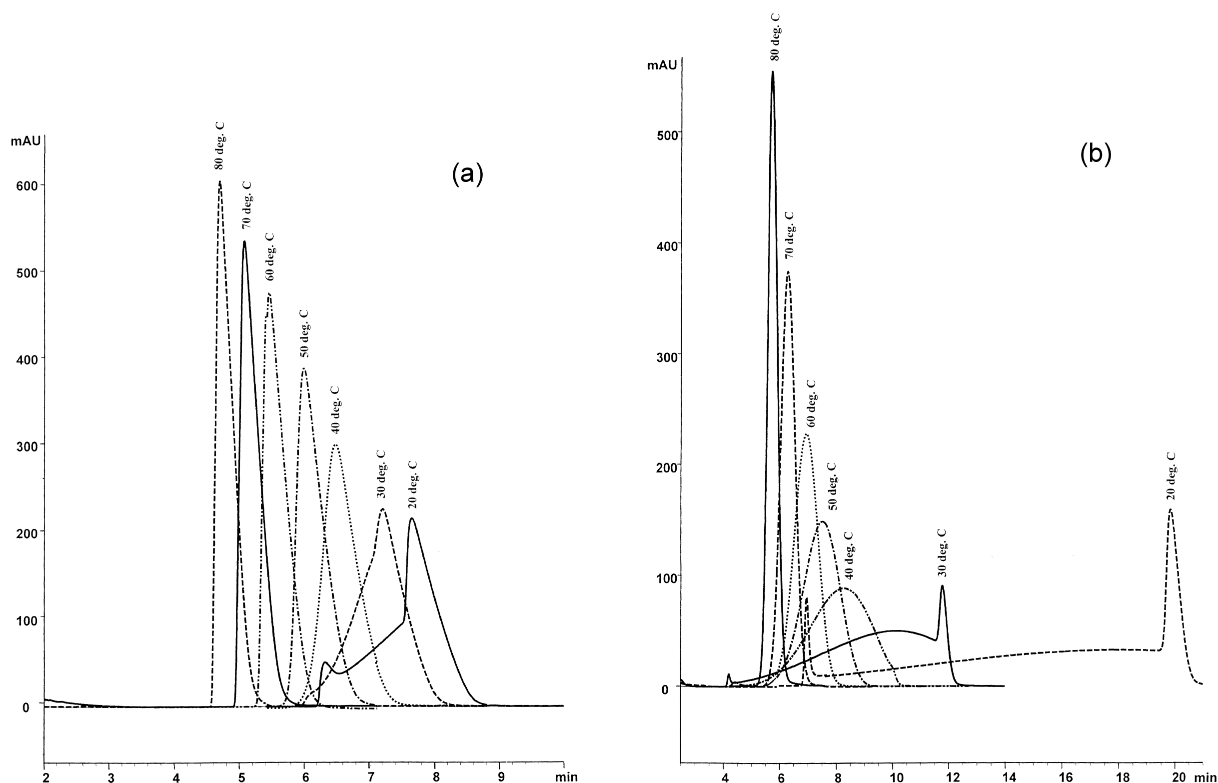


Fig. 3. (a) Chromatograms of enalapril (pH=2) at various column temperatures, (b) Chromatograms of enalapril (pH=7) at various column temperatures.

around the peptide bond and decreases the rate of isomerization. The result is peak broadening and peak splitting. With a neutral mobile phase noticeable rotamer separation is achieved even at room temperature. Using the acidic mobile phase enalapril elutes as a single peak from 80 to 40°C. Below this temperature peak splitting was observed.

A mobile phase of pH 2.0 was chosen for further investigation of enalaprilat, lisinopril and DKP. The corresponding elution profiles for these compounds are given in Fig. 4a–c. The mobile phase consisted of 14% acetonitrile for analysis of the first two drugs and of 40% acetonitrile for the analysis of DKP. At pH 2.0 all three molecules are positively charged [4]. It is interesting to note that the peak shape of DKP is not seriously affected by temperature. The reason lies in the cyclic structure of DKP which prevents rotation. But the other two compounds show a considerable peak-shape change with increasing temperature. By decreasing the temperature from 80°C to room temperature, the chromatographic peak of lisinopril starts to split at 40°C, while the peak of enalaprilat at that temperature is the broadest and starts to split at room temperature.

When analysing enalapril, enalaprilat and DKP in mixture the retention time shortened with increasing temperature. Nevertheless the resolution between those compounds improves, because the chromatographic peaks become narrower, especially for enalapril and enalaprilat, due to the higher rotation around the peptide bond. Since enalapril and DKP are much less polar then enalaprilat, gradient analysis is usually used. In the presented study different amounts of acetonitrile (14%, 32% and 40%) in mobile phase were chosen to obtain similar retention factors for studied compounds at 80°C.

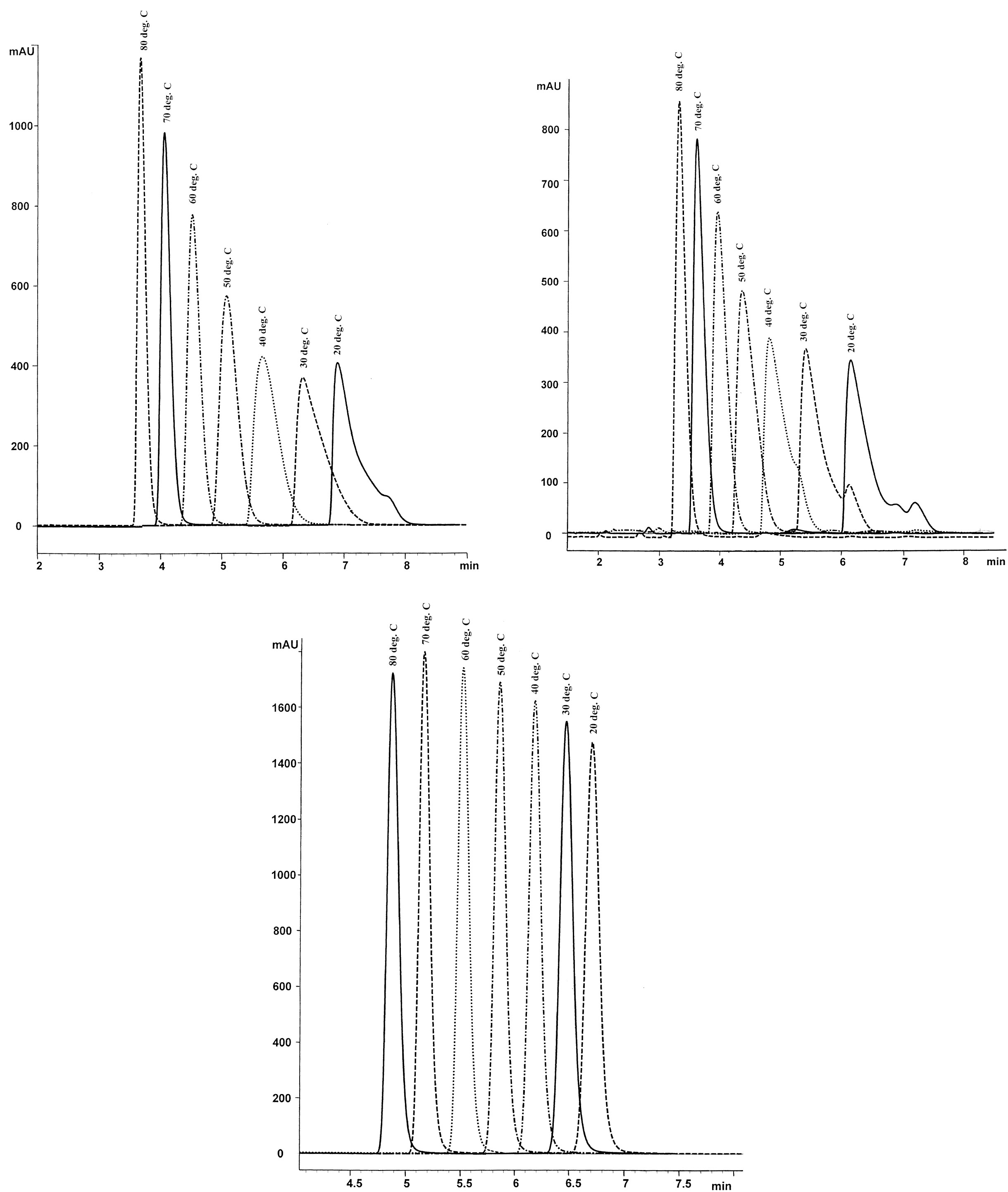


Fig. 4. (a) Chromatograms of enalaprilat (pH=2) at various column temperatures, (b) Chromatograms of lisinopril (pH=2) at various column temperatures, (c) Chromatograms of enalapril-DKP (pH=2) at various column temperatures.

3.2. Isolation of enalapril rotation isomers

To confirm the origin of the split peaks, enalapril maleate *cis*- and *trans*-rotation isomers were isolated by analytical HPLC. Separation was achieved on an analytical column (250×4.6) mm packed with 5 μm Spherisorb Octyl. Twenty m*M* phosphate buffer pH 7.0 containing 17% acetonitrile was used as mobile phase. Column and mobile phase were thermostated at 6°C. 20 μl of enalapril solution (50 mg/ml) was injected. Two fractions were collected in vials and immediately stored in liquid nitrogen. Preparative chromatogram is shown in Fig. 5. First chromatographic peak eluting at 1.2 min is a maleic acid. To confirm the purity of the isolated isomers, the two fractions were reanalysed (under the same conditions as during their isolation). The chromatographic purity of fraction 1 was 88.7% and of fraction 2 92.6% respectively. It can be seen on the basis of the Ala-Pro molecule structure [9] (Fig. 2), that the *cis*-conformer has a larger hydrophobic surface and therefore interacts strongly with C8 ligands on the stationary phase, and thus we can predict that fraction 1 is the *trans*- and fraction 2 the *cis*-rotamer.

3.3. NMR measurements

^{1}H NMR spectrum of enalapril was obtained by the measurement of the sample at the room temperature when both rotamers are present.

^{1}H NMR assignment of enalapril in ppm at room temperature with reference to external TMS (0 ppm); most of the peaks are overlapping multiplets due to the rotamer chemical shifts differences: 7.30–7.50 (5H, Phe), 4.8 (residual HOD), 4.15–4.35 (2H, O$\mathbf{CH_2}$$CH_3$; *, N-CH Pro; **, N-CH Pro), 3.60–3.47 (*, CH Ala; **,CH Ala; 2H, NCH_2 Pro), 3.45 (*, CH Phe), 3.26 (**, CH Phe), 2.70–2.90 (2H, Phe$\mathbf{CH_2}$$CH_2$), 1.80–2.40 (4H, CH_2 Pro; 2H, Phe$CH_2$$\mathbf{CH_2}$), 1.34 (3H, *t*, J=7.2 Hz, $CH_2$$\mathbf{CH_3}$ Et), 1.32 (*, *d*, J=6.90 Hz, CH_3 Ala), 1.30 (**, *d*, J=6.90 Hz, CH_3 Ala),*,** assign distinct rotamer peaks of the same group.

For confirmation of the identity of the isolated rotamers, ^{1}H NMR measurements were carried out at a probe temperature of 6°C. Deuterium oxide (99.9% atom % D) was added to isolated fractions. Sample prepared for NMR measurements consisted of 30 vol% of deuterium oxide. Solvents peaks of HOD

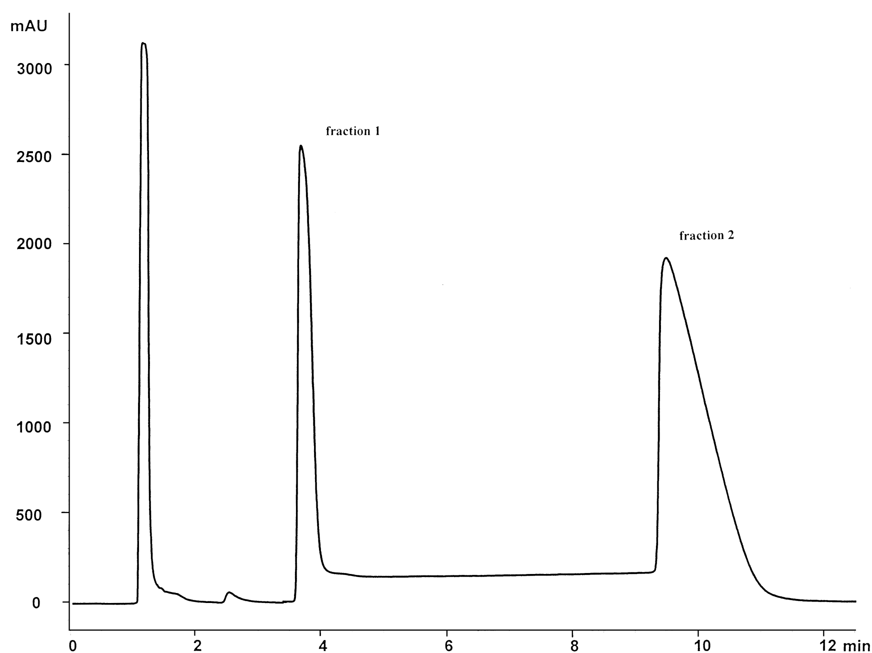


Fig. 5. Preparative chromatogram of enalapril maleate at 6°C column temperature.

and acetonitrile were suppressed by presaturation during a delay time of 2 s.

The ^{1}H NMR spectrum at the beginning of the measurement ($t=0$) shows single signals for the methyl groups, a triplet (1.11 ppm) and a doublet (1.08 ppm). The appearance of a novel doublet signal at 1.06 ppm after 30 min and 2 h of measurement indicates that the process of reequilibration takes place even at the temperature of 6°C and that the isolated fractions were pure isomers. It can be noticed that the chemical shifts are temperature dependent.

4. Conclusion

Chromatographic conditions such as column temperature and mobile phase pH have a dramatic effect on the chromatographic behaviour of enalapril, enalaprilat and lisinopril. The slow rate of the interconversion of rotamers, comparable with the elution velocity on the chromatographic column, induces peak broadening and peak splitting. ACE-inhibitors elute as a single sharp peak at high column temperature (80°C) and low pH (pH=2). On the other hand, some rotamers (enalapril) can be separated at a low column temperature using a neutral mobile phase. The chromatographic peak of DKP is not temperature dependent, because of its cyclic structure. By lowering the column temperature to 6°C complete separation of *cis*- and *trans*-isomers of enalapril was achieved. To confirm the structure of both isomers a higher quantity of enalapril was separated, and in the isolated fractions different rotation isomers were identified by 1H NMR spectroscopy.

For the determination of studied compounds in mixture high column temperature (60°C) and gradient analysis is recommended by pharmacopoeia [15] to avoid peak splitting caused by rotamers interconversion.

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