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Multiple peak formation in reversed-phase liquid chromatography of ramipril and ramiprilate

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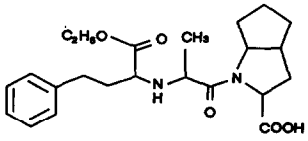
SUMMARY

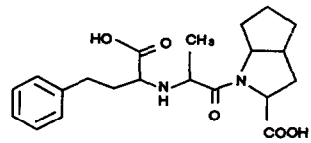
The chromatographic performance of ramipril, an angiotensin-converting enzyme inhibitor, and ramiprilate, its active metabolite, was studied on an octadecyl-bonded silica stationary phase and with aqueous eluents. The substances are peptide-related with a proline residue, a group of compounds known to exist as a mixture of two conformers in aqueous solution. The influence of column temperature, flow-rate, pH, organic modifier and stationary phase was investigated, and the *cis* and *trans* isomers of ramiprilate were assigned by nuclear magnetic resonance studies. Multiple and irregularly shaped chromatographic peaks were obtained, but by choosing appropriate conditions the compounds were eluted as single, symmetric peaks, or the two isomers of each compound could be separated.

INTRODUCTION

Multiple peak formation in reversed-phase liquid chromatography (RP-LC) of two peptide-related drugs, captopril[1] and enalapril[2], used as angiotensin-converting enzyme (ACE) inhibitors, and a tridecapeptide[3], all containing a proline residue, have been observed. Horváth and co-workers[4,5] reported that dipeptides containing L-proline may show peak splitting in RP-LC, owing to slow *cis–trans* isomerization. This is caused by hindered rotation around the N-substituted peptide bond. Similar observations in RP-LC have also been made for a new ACE inhibitor, ramipril (a drug developed by Hoechst), and its active metabolite ramiprilate[6].



ramipril



ramiprilate

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In this work, we studied the influence of various operating conditions on the retention, peak splitting and band broadening of ramipril and ramiprilate. The *cis* and *trans* isomers of ramiprilate were assigned by nuclear magnetic resonance (NMR) studies. Eluate fractions containing each of the chromatographically resolved isomers of ramiprilate were subjected to enzyme kinetic studies. These, and extended NMR studies, will be reported elsewhere.

EXPERIMENTAL

Chromatography

The liquid chromatograph consisted of a Model 2150 pump (LKB, Bromma, Sweden), an ISS-100 automatic injector (Perkin-Elmer, Überlingen, F.R.G.), a Model 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.) and a Spectraflow 783 UV detector (Kratos, Ramsey, NJ, U.S.A.). Ramipril and ramiprilate were kindly supplied by Hoechst (Frankfurt am Main, F.R.G.). Buffer substances and acids of analytical-reagent grade were purchased from E. Merck (Darmstadt, F.R.G.) and organic solvents of HPLC grade from Rathburn (Walkerburn, U.K.).

A Nucleosil C_{18} (3 μm) column (100 × 4.6 mm I.D.) (Macherey, Nagel & Co., Düren, F.R.G.) was used and the mobile phase was, unless stated otherwise, phosphate buffer of pH 2.0 ($I = 0.10$) containing various amounts of acetonitrile, tetrahydrofuran (THF) or methanol as organic modifier. Column temperatures above 25°C were controlled with a Model LC-22A column oven (Bioanalytical Systems, West Lafayette, IN, U.S.A.), and for temperatures below 25°C a thermostated water-jacket was used. A 50-μl volume of a sample solution, 150–300 μmol/l in phosphate buffer of pH 2.0, was injected, and the column effluent was monitored at 220 nm.

NMR

The NMR spectra were recorded on a Bruker AM 500 spectrometer. The measurements were performed at ambient temperature both in deuterated dimethyl sulphoxide ([$^2H_6$]DMSO), in order to make a comparison with investigations on related compounds[7], and in [$^2H_2$]water–[$^2H_3$]acetonitrile (80:20), a solution similar to the mobile phase in the chromatographic studies. High-temperature measurements (95°C) were performed in 10 mmol/l phosphate buffer (pH 7.3). The samples were 10 mmol/l with respect to ramiprilate. Two-dimensional (2D) ^1H–^1H and ^1H–^{13}C NMR studies were also undertaken to aid in the assignments.

RESULTS AND DISCUSSION

Chromatographic studies

Column temperature. Chromatograms of ramipril and ramiprilate obtained at different column temperatures, with a mobile phase containing 30% acetonitrile, are shown in Fig. 1. With increasing temperature the conversions between the two rotamers accelerate and single peaks were obtained, at a lower temperature for ramipril than for ramiprilate. Similar effects have been reported in the RP-LC of other proline-containing substances[3–5,8]. The influence of temperature on retention was small for both compounds.

Flow-rate. The influence of the residence time in the column on the peak shape

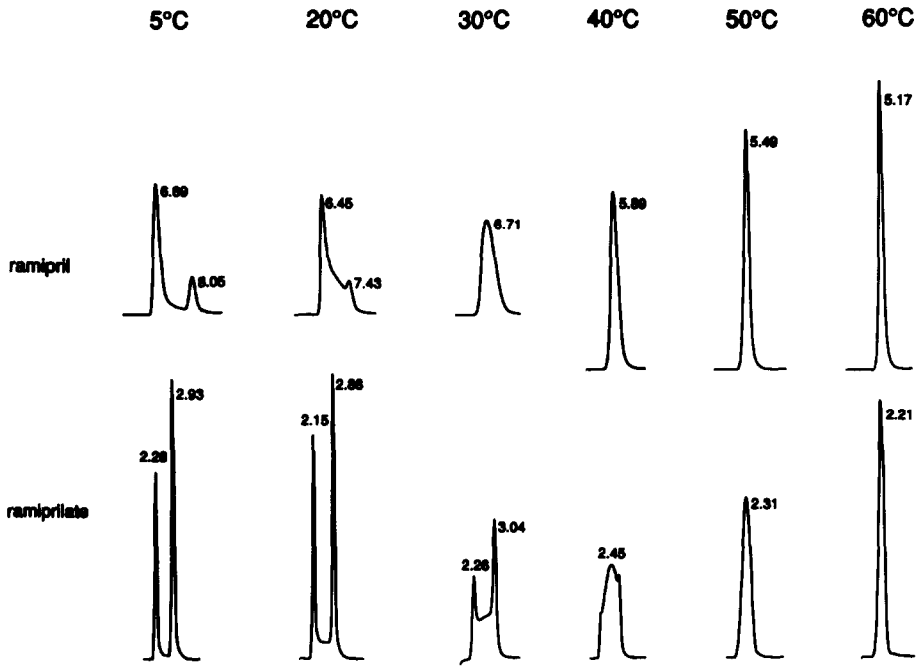


Fig. 1. Effect of column temperature on the chromatographic performance of ramipril and ramiprilate. Stationary phase, Nucleosil C_{18}, 3 μm (100 × 4.6 mm I.D.). Mobile phase, 30% acetonitrile in phosphate buffer (pH 2.0). Flow-rate, 1.0 ml/min. The retention times of the peaks are indicated.

is illustrated in Fig. 2 for ramiprilate, eluted at a flow-rate of 1.0, 0.5 or 0.3 ml/min. A mobile phase containing 30% acetonitrile was used and the column temperature was 30°C. With decreasing flow-rate a more monodisperse peak was obtained. This was also seen for ramipril (not shown here) and has been reported by Horváth and co-

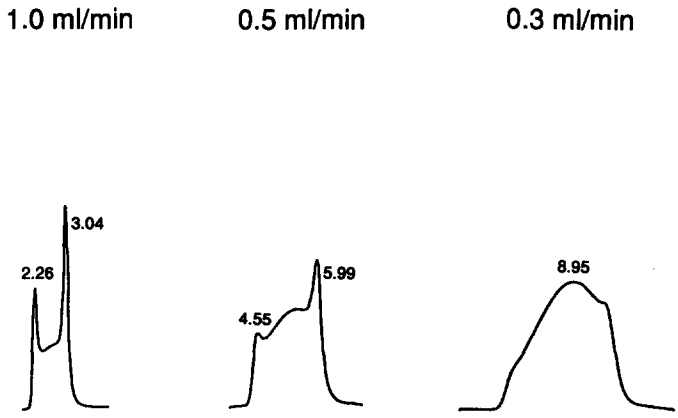


Fig. 2. Effect of flow-rate on the peak shape of ramiprilate. Flow-rate, 1.0, 0.5 or 0.3 ml/min. Column temperature, 30°C. Other conditions as in Fig. 1.

workers[4,5] for proline-containing peptides having relaxation times of *cis–trans* isomerization commensurate with their retention times.

Influence of pH. The pH of the mobile phase had a considerable effect on the chromatographic performance of both ramipril and ramiprilate. The chromatographic peaks obtained at pH 2.0 and 6.0 are shown in Fig. 3. The mobile phase was phosphate buffer containing 25% acetonitrile and 5% THF, and the column temperature was 15°C. The flow-rate at pH 6.0 was 0.5 ml/min in order to give retention times comparable to those at pH 2.0 (1.0 ml/min). The peak shape of ramipril was improved when the pH was decreased, whereas the opposite was found for ramiprilate. A low pH was used to obtain acceptable peak shapes for captopril[1] and enalapril[2], both structurally related to ramipril. For alanylproline a similar effect with decrease in pH was explained by a higher isomerization rate[4].

The influence of pH on the capacity factors (k') of ramipril and ramiprilate is shown in Fig. 4. The mobile phase was phosphate buffer (pH 2 or 3) or acetate buffer (pH 4, 5 or 6) with 20% acetonitrile and 5% THF. A column temperature of 60°C was used to obtain single peaks for both compounds. Ramipril, having acid dissociation

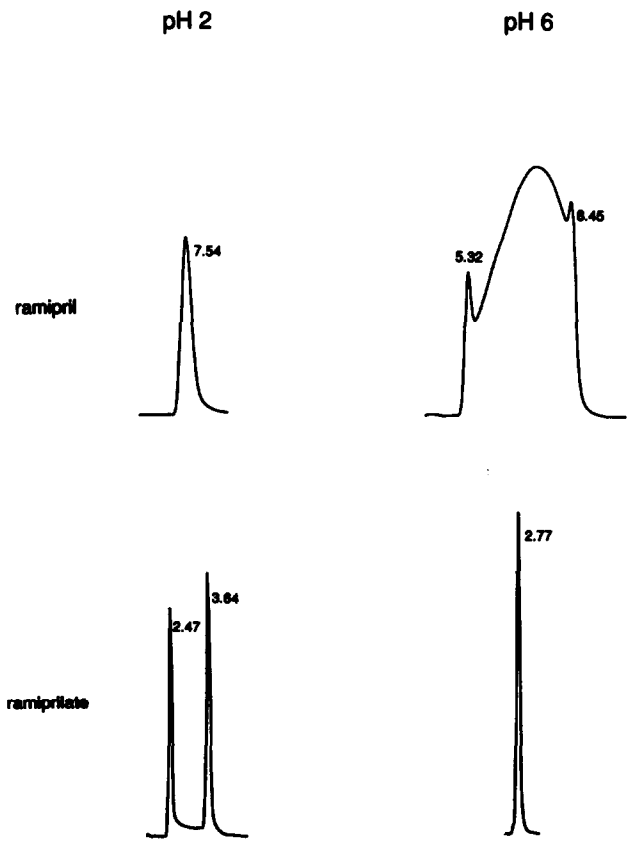


Fig. 3. Effect of pH on the peak shapes of ramipril and ramiprilate. Mobile phase, 25% acetonitrile and 5% THF in phosphate buffer (pH 2.0 or 6.0). Flow-rate, 1.0 ml/min (pH 2) or 0.5 ml/min (pH 6). Column temperature, 15°C. Stationary phase as in Fig. 1.

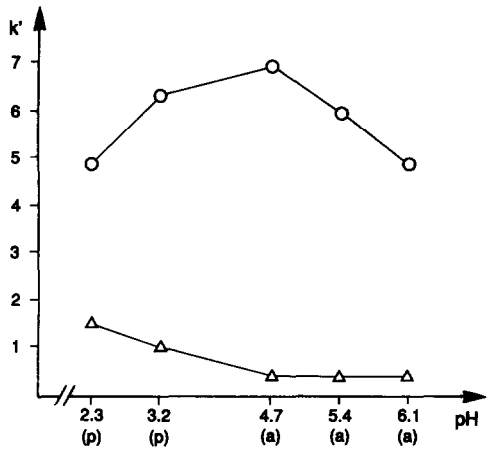


Fig. 4. Influence of pH on the capacity factors (k') of (○) ramipril and (△) ramiprilate. Mobile phase, 20% acetonitrile and 5% THF in (p) phosphate or (a) acetate buffer ($I=0.10$). Column temperature, 60°C. Stationary phase and flow-rate as in Fig. 1.

constants (pK_a) of about 3 and 5.5[6], is zwitterionic with maximum retention in the pH region between the pK_a values. For ramiprilate, having a lowest pK_a of about 1.5 (ref. 6), no retention maximum was obtained under the chromatographic conditions used.

Organic modifier. The nature of the organic modifier in the mobile phase greatly affected the chromatographic performance of ramipril. This is shown in Fig. 5, where

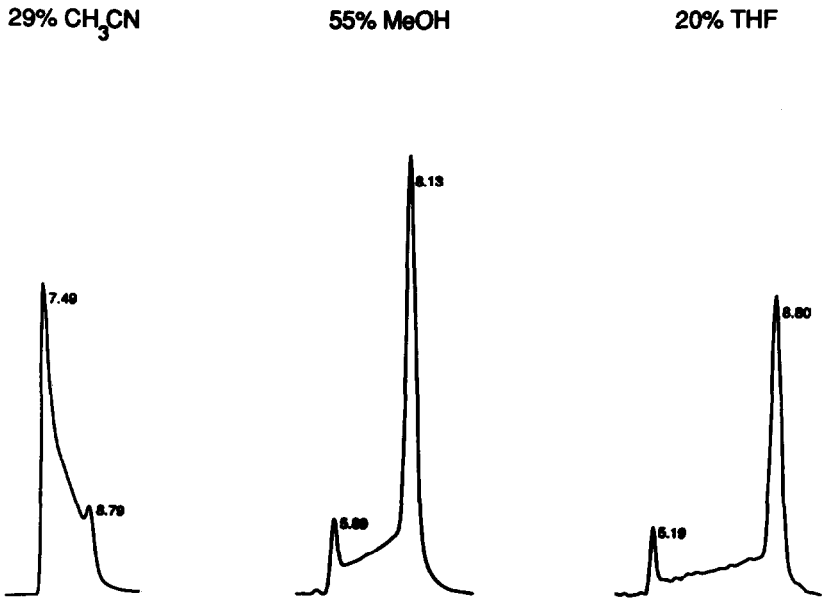


Fig. 5. Effect of organic modifier on the chromatography of ramipril. Mobile phase containing 29% acetonitrile, 55% methanol or 20% THF. Column temperature, 20°C. Other conditions as in Fig. 1. MeOH = Methanol.

acetonitrile, methanol or THF was used as a modifier in aqueous phosphate buffer. The column temperature was 20°C. Different amounts of the modifiers were added to the various systems in order to give comparable retention times. The elution order of the isomers was inverted in the chromatographic systems containing methanol and THF compared with the acetonitrile-containing system. This was verified by collecting fractions of the eluate containing the isomers of ramipril from the acetonitrile system and reinjecting them into the methanol and THF systems. The column was refrigerated at 2°C to give good resolution between the two peaks and the eluate fractions were immediately frozen to prevent transformations between the two conformers.

An improvement in the chromatography of ramipril was obtained by the addition of THF or methanol to a mobile phase containing acetonitrile. Ramipril eluted

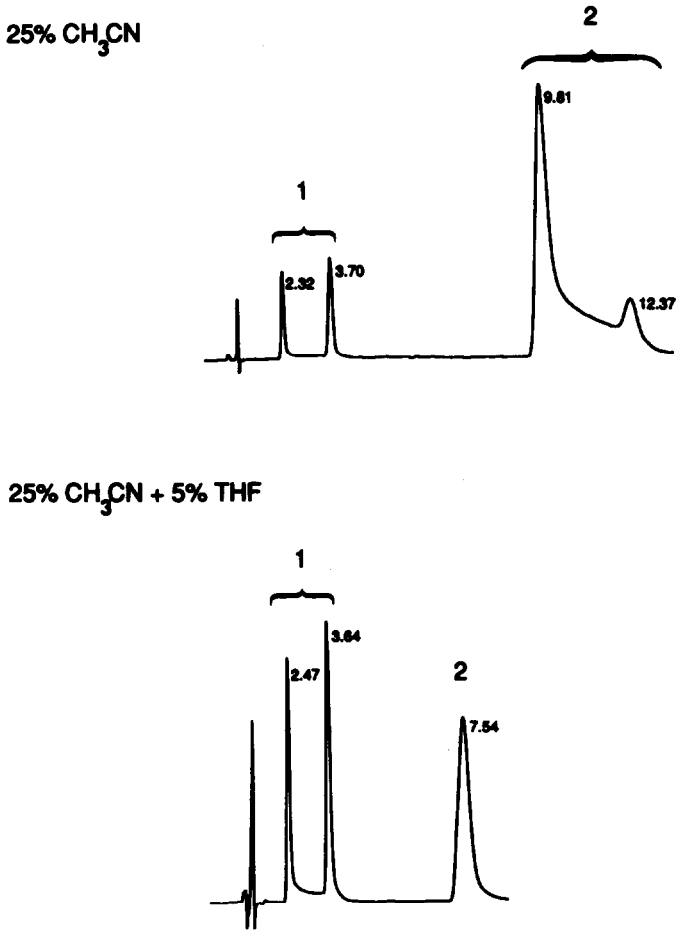


Fig. 6. Effect of addition of THF to an acetonitrile-containing system on the peak shapes of (1) ramiprilate and (2) ramipril. Mobile phase, 25% acetonitrile or 25% acetonitrile and 5% THF in phosphate buffer (pH 2.0) with a flow-rate of 1.5 or 1.0 ml/min, respectively. Column temperature, 15°C. Different attenuations were used.

as a single peak at 15°C when 5% THF was added to a mobile phase containing 25% acetonitrile, as illustrated in Fig. 6, which also shows a chromatogram obtained without THF. Ramiprilate was only slightly affected and eluted as a well resolved doublet in both systems.

Stationary phase. Chromatograms from a cation-exchange column (Nucleosil 5SA, 100 × 4.6 mm I.D.) and a C_{18} column (see Experimental) obtained at 30 and 60°C are shown in Fig. 7. The mobile phase in both chromatographic systems was phosphate buffer (pH 2.0) with 30% acetonitrile. The numbers of theoretical plates (N) for the peaks obtained at 60°C are shown. The C_{18} column was more efficient than the cation exchanger for ramipril, whereas ramiprilate showed the best performance on the cation-exchange column. The elution of ramipril as a bimodal, broad peak on the cation-exchange column at 30°C may be due to a difference in acidity for the *cis* and *trans* rotamers. Such a difference would affect the chromatography on the

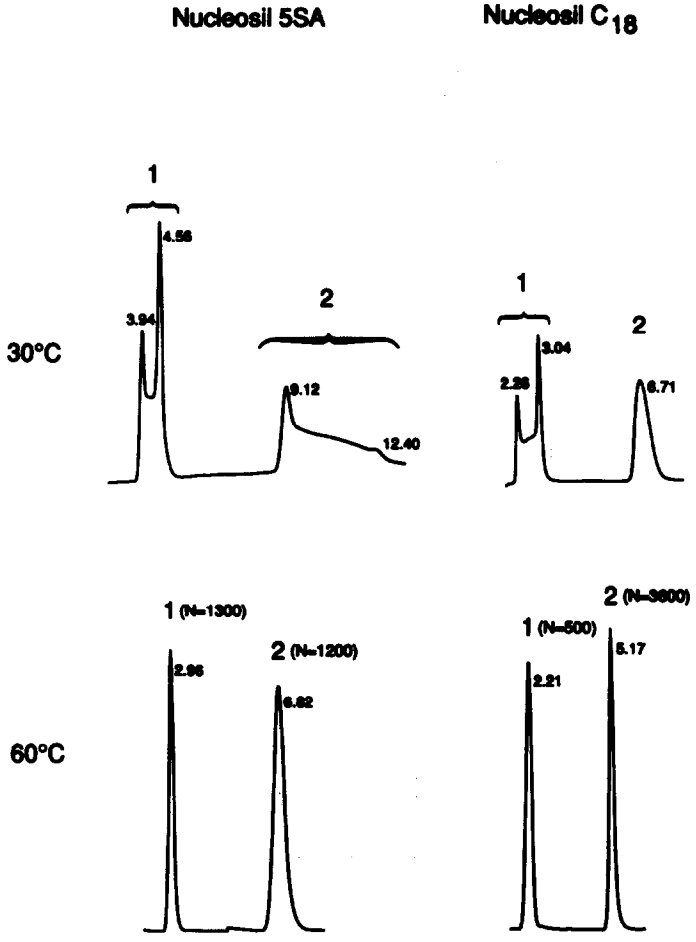
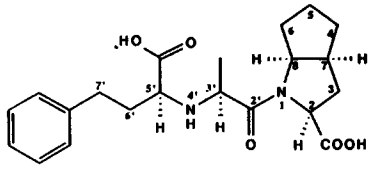


Fig. 7. Chromatograms of (1) ramiprilate and (2) ramipril from a cation-exchange column (Nucleosil 5SA, 100 × 4.6 mm I.D.) and a C_{18} column (the same as in Fig. 1). Column temperature, 30 or 60°C. Other conditions as in Fig. 1. The numbers of theoretical plates (N) at 60°C are shown.

cation exchanger more than it would on the C_{18} column, from which a single peak was obtained. Different pK_a values for the isomers of some proline- and sarcosine-containing peptides have been determined by NMR[9,10]. The results indicated intramolecular hydrogen bonding, which stabilizes the protonated *trans* isomer and decreases its acidity.

NMR studies

In Fig. 8, the ^1H NMR spectrum of ramiprilate in [$^2H_6$]DMSO is shown. In the chemical shift range 3–4.6 ppm the spectrum exhibits two distinct sets of signals for each proton, which reveals the existence of two contributing conformers. These rotamers are assigned to the *cis–trans* equilibrium of the rotation around the amide bond. No assignment was made of signals in the region 1–3 ppm owing to difficulties with overlapping. 2D NMR studies clearly showed, however, that more than a single set of resonances contribute to the spectrum. The rotamer ratio in DMSO was integrated to be 65:35, which is similar to the ratio of the areas of the two peaks obtained after



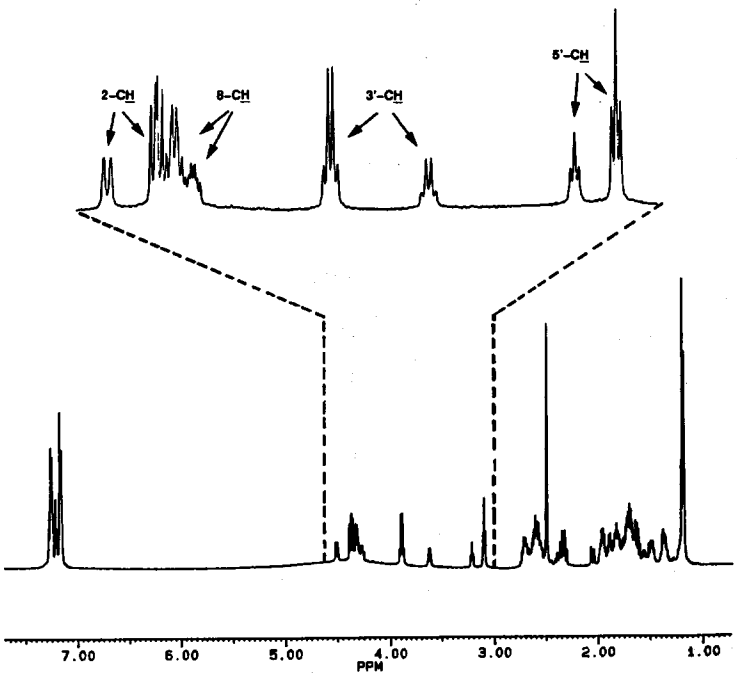


Fig. 8. ^1H NMR spectrum of ramiprilate (5 mg in 0.6 ml) in [$^2H_6$]DMSO.

injection of ramiprilate in DMSO into the refrigerated chromatographic system containing acetonitrile. In [$^2H_2$]water–[$^2H_3$]acetonitrile the ratio was integrated to be 53:47, also in good agreement with chromatographic results. This confirms that the ratio of the two conformers, chromatographed on a cooled column, is entirely dependent on the sample conditions prior to injection. Based on the 2D NMR studies and on comparison with studies on related compounds', the major rotamer was assigned to the *trans* form. At high temperature (95°C) the NMR spectra exhibited unambiguous exchange broadening. Line-shape analysis according to McConnell' [1] gave a barrier for *trans* to *cis* rotation of 85 kJ/mol, which is of a magnitude that explains the peak splitting and band broadening seen in the chromatographic studies.

CONCLUSION

Various operating conditions greatly affected the anomalous chromatographic behaviour of the proline-containing substances ramipril and ramiprilate. The compounds eluted as increasingly monodisperse peaks with decreasing flow-rate, and with increasing column temperature single peaks were obtained. The peak shape of ramiprilate was improved when the pH was increased or if the reversed-phase column was replaced by a cation exchanger, while the opposite was found for ramipril. The elution order and the appearance of the isomers of ramipril were dependent on the nature of the organic modifier. By choosing appropriate conditions, the compounds were either eluted as single peaks or the isomers of each compound could be separated. The first-eluting isomer of ramiprilate in the chromatographic system containing acetonitrile was characterized by NMR studies as the *tram* rotamer.

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